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November 11, 2003

American
Chemistry
Council

Charlie Auer
Director
TSCA Document Control Office (7407)
EPA East Building, Room 6428
Office of Pollution Prevention and Toxics
U.S. Environmental Protection Agency
1201 Constitution Avenue, N.W.
Washington, DC 20460-0001

Contain NO CBI

Dear Mr. Auer:

The American Chemistry Council (Council) makes available to the public and appropriate government agencies final reports of environmental, health, and safety research that it manages. In keeping with this policy, the following seven final reports that the Council's Brominated Flame Retardant Industry Panel (BFRIP) recently conducted are enclosed:

- Tetrabromobisphenol A: A 48-Hour Flow-Through Toxicity Test with the Cladoceran (*Daphnia magna*);
- Tetrabromobisphenol A: A 96-Hour Flow-Through Acute Toxicity Test with the Rainbow Trout (*Oncorhynchus mykiss*);
- Hexabromocyclododecane (HBCD): An Activated Sludge, Respiration Inhibition Test;
- Hexabromocyclododecane (HBCD): A Prolonged Sediment Toxicity Test with *Hyalella azteca* Using Spiked Sediment with 2% Total Organic Carbon;
- Hexabromocyclododecane (HBCD): A Prolonged Sediment Toxicity Test with *Hyalella azteca* Using Spiked Sediment with 5% Total Organic Carbon;
- Hexabromocyclododecane: Contact Sensitization Potential via the Local Lymph Node Assay (Including a Primary Irritancy Screen) Using CBA/J Mice; and,
- Evaluation of Aerobic and Anaerobic Transformation of Hexabromocyclododecane in Soil.

These reports do not include confidential information.

If you have any questions, please contact Wendy K. Sherman, the BFRIP Manager, at 703/741-5639 or via email at wendy_sherman@americanchemistry.com.

Sincerely yours,

Wendy K. Sherman

Wendy K. Sherman
Director, CHEMSTAR



Enclosures (7)



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TETRABROMOBISPHENOL A:
A 48-HOUR FLOW-THROUGH ACUTE TOXICITY TEST
WITH THE CLADOCERAN (*Daphnia magna*)

FINAL REPORT

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER : 439A-124

OECD GUIDELINE 202

and

U.S. EPA OPPTS NUMBER 850.1010

AUTHORS :

Amy S. Blankinship, M.S.
Raymond L Van Hoven, Ph.D.
Henry O. Krueger, Ph.D.

STUDY INITIATION DATE: February 28, 2003

STUDY COMPLETION DATE: July 8, 2003

SUBMITTED TO :

American Chemistry Council's
Brominated Flame Retardant Industry Panel
1300 Wilson Boulevard
Arlington, Virginia 22209

Wildlife International, Ltd.

8598 Commerce Drive
Easton, Maryland 21601
(410) 822-8600

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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

SPONSOR: American Chemistry Council's Brominated Flame Retardant Industry Panel


TITLE: Tetrabromobisphenol A: A 48-Hour Flow-Through Acute Toxicity Test with the Cladoceran (*Daphnia magna*)

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 439A-124

STUDY COMPLETION: July 8, 2003

This study was conducted in compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency in 40 CFR Parts 160 and/or 792, 17 August 1989; OECD Principles of Good Laboratory Practice (ENV/MC/CHEM (98)17); and Japan MAFF, 11 NohSan, Notification No. 6283, Agricultural Production Bureau, 1 October 1999.

STUDY DIRECTOR:



Amy S. Blankinship, M.S.
Aquatic Biologist

7-8-03
Date

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QUALITY ASSURANCE STATEMENT

This study was examined for compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency in 40 CFR Parts 160 and/or 792, 17 August 1989; OECD Principles of Good Laboratory Practice (ENV/MC/CHEM (98)17); and Japan MAFF, 11 NohSan, Notification No. 6283, Agricultural Production Bureau, 1 October 1999. The dates of all inspections and audits and the dates that any findings were reported to the Study Director and Laboratory Management were as follows:

ACTIVITY:	DATE CONDUCTED:	DATE REPORTED TO:	
		STUDY DIRECTOR:	MANAGEMENT:
Protocol	March 4, 2003	March 4, 2003	March 7, 2003
Test Substance Preparation	March 4, 2003	March 5, 2003	March 5, 2003
Matrix Fortification	March 5, 2003	March 5, 2003	March 7, 2003
Environmental Conditions, Sample Collection, and Water Chemistry	March 7, 2003	March 7, 2003	March 7, 2003
Biological Data and Draft Report	March 19, 2003	March 19, 2003	March 20, 2003
Analytical Data and Draft Report	March 19, 2003	March 19, 2003	March 20, 2003
Final Report	July 2, 2003	July 2, 2003	July 3, 2003

All inspections were study-based unless otherwise noted.

Susan L. Coleman
Susan L. Coleman, B.A.
Senior Quality Assurance Representative

7-8-03
Date

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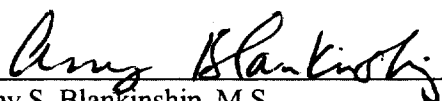
REPORT APPROVAL

SPONSOR: American Chemistry Council's Brominated Flame Retardant Industry Panel

TITLE: Tetrabromobisphenol A: A 48-Hour Flow-Through Acute Toxicity Test with the Cladoceran (*Daphnia magna*)

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 439A-124

STUDY DIRECTOR:

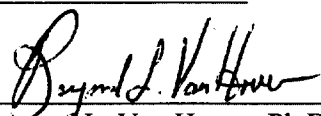


Amy S. Blankinship, M.S.
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Raymond L. Van Hoven, Ph.D.
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Willard B. Nixon, Ph.D.
Director of Chemistry

7/8/03

Date

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SUMMARY

SPONSOR: American Chemistry Council's Brominated Flame Retardant Industry Panel

TITLE: Tetrabromobisphenol A : A 48-Hour Flow-Through Acute Toxicity Test with the Cladoceran (*Daphnia magna*)

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 439A-124

TEST DATES: Experimental Start (OECD): March 4, 2003
 Experimental Start (EPA): March 5, 2003
 Biological Termination: March 7, 2003
 Experimental Termination: March 7, 2003

LENGTH OF EXPOSURE: 48 Hours; Flow-Through Conditions

TEST ORGANISMS: Cladoceran (*Daphnia magna*)

SOURCE OF TEST ORGANISMS: Wildlife International, Ltd. Cultures
 Easton, Maryland 21601

AGE OF TEST ORGANISMS: Neonates <24 hours at test start

TEST CONCENTRATIONS:	<u>Nominal</u>	<u>Mean Measured</u>
	Negative Control	<LOQ
	Solvent Control	<LOQ
	1.2 mg a.i./L	1.2 mg a.i./L
	1.8 mg a.i./L	1.8 mg a.i./L

RESULTS (Based on Mean Measured Concentrations):

48-Hour EC50:	> 1.8 mg a.i./L
95% Confidence Interval :	Not Applicable
No Mortality/Immobility Concentration:	1.8 mg a.i./L

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INTRODUCTION

This study was conducted by Wildlife International, Ltd. for American Chemistry Council's Brominated Flame Retardant Industry Panel at the Wildlife International, Ltd. aquatic toxicology facility in Easton, Maryland. The definitive toxicity test was conducted from March 5 to 7, 2003. Raw data generated by Wildlife International, Ltd. and a copy of the final report are filed under Project Number 439A-124 in archives located on the Wildlife International, Ltd. site.

OBJECTIVE

The objective of this study was to determine if the acute toxicity of Tetrabromobisphenol A (TBBPA) to the cladoceran, *Daphnia magna*, during a 48-hour exposure period under flow-through test conditions would be > 1.0 mg active ingredient (a.i.)/L.

EXPERIMENTAL DESIGN

Daphnids were exposed to two test concentrations, a negative control (dilution water), and a solvent control (0.1 mL dimethyl formamide/L). Two replicate test chambers were maintained in each treatment and control group, with 10 daphnids in each test chamber for a total of 20 daphnids per concentration. Two nominal test concentrations were selected by the Sponsor to be reasonably assured that at least one mean measured concentration would be > 1.0 mg a.i./L. Nominal test concentrations selected were 1.2 and 1.8 mg a.i./L. Mean measured test concentrations were determined from samples of test water collected from each treatment and control group at test initiation and termination.

Delivery of the test substance was initiated approximately 17 hours prior to test initiation in order to achieve equilibrium of the test substance in the test chambers. Daphnids were impartially assigned to exposure chambers at test initiation. Observations of mortality, immobility and other signs of toxicity were made approximately 4.5, 24 and 48 hours after test initiation. Cumulative percent mortality and immobility observed in the treatment groups were used to estimate EC50 values at 24 and 48 hours. The no-mortality/immobility concentration was determined by visual interpretation of the mortality and immobility data.

MATERIALS AND METHODS

The study was conducted based on the procedures outlined in the protocol, "Tetrabromobisphenol A: A 48-Hour Flow-Through Acute Toxicity Test with the Cladoceran (*Daphnia magna*)". The protocol was based on procedures outlined in OECD Guidelines for Testing of Chemicals, 202: *Daphnia sp. Acute Immobilization Test and Reproduction Test* (1); U.S. Environmental Protection Agency Series 850 – Ecological Effects Test Guidelines (draft) OPPTS Number 850.1010, *Aquatic Invertebrate Acute Toxicity Test, Freshwater Daphnids* (2); and ASTM Standard E729-88a, *Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates and Amphibians* (3).

Test Substance

The test substance used in the study consisted of a composite of TBBPA samples received from three manufacturers (Great Lakes Chemical Corporation (lot# 1008JE04B), Albermarle Corporation (lot# 25115T-1), and Bromine Compounds, Ltd. (lot# 010040)) between August 16 – 31, 2001. The composite sample was prepared by Wildlife International, Ltd. and assigned Wildlife International identification number 5754. Subsamples of the composite were shipped to Albermarle Corporation for characterization and purity analysis. The test substance, a white powder, was identified as: TBBPA composite; CAS Number 79-94-7. The reported purity was 99.17%, but the sponsor did not indicate an expiration date. The test substance was stored under ambient conditions.

Test Organism

The cladoceran, *Daphnia magna*, was selected as the test species for this study. Daphnids are representative of an important group of aquatic invertebrates and were selected for use in the test based upon past history of use and ease of culturing in the laboratory. Daphnid neonates used in the test were less than 24-hours old and were obtained from cultures maintained by Wildlife International, Ltd., Easton, Maryland.

Adult daphnids were cultured in water from the same source and at approximately the same temperature as used during the test. The adult daphnids used to supply neonates for the test were held for 22 days prior to collection of the juveniles for testing. The adults showed no signs of disease or stress during the holding period. During the 2-week holding period immediately preceding the test, water temperatures ranged from 19.8 to 21.0°C, measured with a hand-held liquid-in-glass

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thermometer. The pH of the water ranged from 8.3 to 9.0, measured with a Fisher Scientific Accumet Model 915 pH meter. Dissolved oxygen ranged from 8.2 to 9.0 mg/L ($\geq 91\%$ of saturation), measured with a Yellow Springs Instruments Model 51B dissolved oxygen meter. Daphnids in the cultures were fed daily a mixture of yeast, Cerophyll[®], and trout chow, as well as a suspension of the freshwater green alga, *Selenastrum capricornutum*. The adults were fed prior to test initiation, but neonates were not fed during the test.

Neonate daphnids were obtained for testing from five individual adult daphnids. At test initiation, the juvenile daphnids were collected from the cultures and indiscriminately transferred one or two at a time to transfer chambers (e.g., 10 mL glass beakers) until each chamber contained ten daphnids. All transfers were made below the water surface using wide-bore pipettes. The transfer chambers were indiscriminately assigned to the test chambers and were placed inside the test compartments. The daphnids were released into the test compartments by gently submerging the compartments in the test solution.

Dilution Water

The water used for culturing and testing was freshwater obtained from a well approximately 40 meters deep located on the Wildlife International, Ltd. site. The well water is characterized as moderately-hard water. The specific conductance, hardness, alkalinity and pH of the well water during the four-week period immediately preceding the test are presented in Appendix 1.

The well water was passed through a sand filter to remove particles greater than approximately 25 μm , and pumped into a 37,800-L storage tank where the water was aerated with spray nozzles. Prior to use, the well water was filtered to 0.45 μm and then passed through an ultraviolet (UV) sterilizer to remove microorganisms and fine particles. The results of periodic analyses performed to measure the concentrations of selected organic and inorganic constituents in the well water used by Wildlife International, Ltd. are presented in Appendix 2.

Test Apparatus

A continuous-flow diluter was used to deliver each concentration of the test substance, a solvent (dimethyl formamide (DMF)) control, and a negative (well water) control. A syringe pump (Harvard Apparatus, South Natick, Massachusetts) was used to deliver the two test substance stock

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solutions and DMF for the solvent control into mixing chambers assigned to each treatment and the solvent control. The syringe pump was calibrated prior to the test. The stock solutions were mixed with dilution water in the mixing chambers in order to obtain the desired test concentrations. The flow of dilution water to the mixing chambers was controlled by rotameters which were calibrated prior to the test. The flow of test water from each mixing chamber was split and allowed to flow into replicate test chambers. The proportion of test water that was split into each replicate was checked prior to the test to ensure that flow rates varied by no more than $\pm 10\%$ of the mean for the two replicates.

The diluter was adjusted so that each test chamber received approximately 13 volume additions of test water every 24 hours. The general operation of the diluter was checked visually at least two times per day during the test and at least once at the beginning and end of the test.

Test chambers were 9-L glass aquaria filled with approximately 7 L of test water. The depth of the test water in a representative chamber was approximately 14.8 cm. Each test chamber contained one test compartment constructed from a glass cylinder approximately 50 mm in diameter, with 425 μm nylon screen attached to the bottom using silicone sealant. The cylinder was inverted inside a 150-mL glass beaker, which was submerged in the test chamber. Test chambers were indiscriminately positioned in a temperature-controlled environmental chamber set to maintain the desired test temperature. The test chambers were labeled with the project number, test concentration and replicate.

Preparation of Test Concentrations

One stock solution was prepared for each of the two concentrations tested. A primary stock solution was prepared in DMF at a concentration of 18 mg a.i./mL. The primary stock solution was mixed by inversion and sonication, and was clear and colorless. An aliquot of the primary stock solution was proportionally diluted with DMF to prepare an additional stock solution at a concentration of 12 mg a.i./mL. The stock solution was mixed by inversion, and was clear and colorless. The two stock solutions were injected into the diluter mixing chambers (at a rate of 12.50 $\mu\text{L}/\text{minute}$) where they were mixed with well water (at a rate of 125 mL/minute) to achieve the desired test concentrations. The solvent control was prepared by injecting DMF only into the mixing chamber for the solvent control. The concentration of DMF in the solvent control and all TBBPA

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treatment groups was 0.1 mL/L. All test solutions appeared clear and colorless in the diluter mixing chambers and in the test chambers at test initiation and termination.

Analytical Sampling

Samples were collected from both replicate test chambers in each treatment and control group at test initiation and termination to measure concentrations of the test substance. All test solution samples were collected at mid-depth, placed in glass vials, and analyzed as soon as possible without storage.

Analytical Method

The analytical method used for the analysis of TBBPA in freshwater was developed at Wildlife International, Ltd. (Appendix 3). The analytical method consisted of dilution of the aqueous samples in 50% (v/v) methanol in NANOpure[®] water solution, and analysis by direct injection high performance liquid chromatography mass spectrometry (HPLC/MS).

Concentrations of TBBPA were determined by HPLC/MS using a Hewlett-Packard Model 1100 High Performance Liquid Chromatograph interfaced with a Perkin-Elmer SCIEX API 100 Mass Spectrometer. Chromatographic separations were achieved with a Keystone Betasil C₁₈ column (50 mm × 2 mm, 3-μm particle size) fitted with a Keystone Javelin C₁₈ guard column (20 mm × 2 mm). A flow chart for the analysis of TBBPA is provided in Appendix 3.1, and typical instrumental parameters are summarized in Appendix 3.2.

Calibration standards of TBBPA, ranging in concentration from 0.0100 to 0.100 mg a.i./L, were prepared in 50% (v/v) methanol in NANOpure[®] water solution using a stock solution of TBBPA in methanol (Appendix 3.3). Linear regression equations were generated using the peak area for each standard versus the respective concentrations of the calibration standards. The concentration of TBBPA in the samples was determined by substituting the peak area responses into the applicable linear regression equation. An example of the calculations for a representative sample is included in Appendix 3.4.

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The limit of quantitation (LOQ) for the freshwater analyses was set at 0.250 mg a.i./L, calculated as the product of the lowest calibration standard (0.0100 mg a.i./L) and the dilution factor of the matrix blank samples (25).

Quality control samples were prepared in freshwater and analyzed concurrently with test samples at each sampling interval to assess the performance of the analytical methodology. Two matrix blank samples were analyzed to determine possible interferences. No interferences were observed at or above the LOQ in the matrix blanks during the sample analyses (Appendix 3.5). Samples of freshwater were fortified at 1.00, 1.50, and 2.00 mg a.i./L using the appropriate fortification stock solution of TBBPA in methanol (Appendix 3.3), and were analyzed concurrently with the samples. The measured concentrations for the matrix fortification samples ranged from 98.8 to 103% of nominal concentrations (Appendix 3.5).

A representative calibration curve for TBBPA is presented in Appendix 3.6. Representative chromatograms of low and high-level calibration standards are presented in Appendices 3.7 and 3.8, respectively. A representative chromatogram of a matrix blank sample is presented in Appendix 3.9, and a representative chromatogram of a matrix fortification sample is presented in Appendix 3.10. A representative chromatogram of a test sample is presented in Appendix 3.11.

Environmental Conditions

Lighting used to illuminate the cultures and test chambers during holding, acclimation and testing was provided by fluorescent tubes that emitted wavelengths similar to natural sunlight (Colortone® 50). A photoperiod of 16 hours of light and 8 hours of darkness was controlled with an automatic timer. A 30-minute transition period of low light intensity was provided when lights went on and off to avoid sudden changes in lighting. Light intensity at test initiation was 479 lux at the surface of the water of one representative test chamber.

The target test temperature during the study was $20 \pm 1^{\circ}\text{C}$. Temperature was measured in each test chamber at the beginning and end of the test using a liquid-in-glass thermometer. Temperature also was measured continuously during the test in one negative control test chamber using a Fulscope ER/C Recorder, which was verified prior to test initiation with a liquid-in-glass thermometer. Dissolved oxygen and pH were measured in alternating test chambers of each

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treatment and control group at the beginning and end of the test, and at approximately 24-hours. Hardness, alkalinity, specific conductance and total organic carbon (TOC) were measured in the dilution water at the beginning of the test.

Light intensity was measured using a SPER Scientific Model 840006C light meter. Dissolved oxygen was measured using a Thermo Orion Model 850Aplus dissolved oxygen meter, and measurements of pH were made using a Thermo Orion Model 525Aplus meter. Specific conductance was measured using a Yellow Springs Instrument Model 33 Salinity-Conductivity-Temperature meter. Hardness and alkalinity measurements were made by titration based on procedures in *Standard Methods for the Examination of Water and Wastewater* (4).

Observations

Observations were made to determine the numbers of dead and immobile organisms. Immobility was defined as a lack of movement by the organism except for minor activity of the appendages. The number of individuals exhibiting signs of toxicity or abnormal behavior also were evaluated. Observations were made approximately 4.5, 24 and 48 hours after test initiation. Prior to test termination, observations were conducted without removing the test compartments from the test solution in order to minimize disturbance of the daphnids. Therefore, the 4.5 and 24-hour observations were estimates, with exact counts of mortality/immobility conducted at test termination.

Statistical Analyses

The absence of mortality or immobility in this study precluded the statistical calculation of EC50 values. Therefore, the 24 and 48-hour EC50 values were estimated to be greater than the highest concentration tested. The no mortality concentration and the no-observed-effect-concentration (NOEC) were determined by visual interpretation of the mortality and observation data.

RESULTS AND DISCUSSION

Measurement of Test Concentrations

Nominal concentrations selected for use in this study were 1.2 and 1.8 mg a.i./L. Results of analyses to measure concentrations of Tetrabromobisphenol A (TBBPA) in the test solution samples collected during the test ranged from 95 to 104% of nominal (Table 1). When the measured concentrations of the test samples collected at test initiation and termination were averaged, the mean

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measured concentrations for the study were 1.2 and 1.8 mg a.i./L, representing 100 and 100% of nominal concentrations, respectively. The results of the study were based on the mean measured test concentrations.

Observations and Measurements

Measurements of temperature, dissolved oxygen and pH of the water in each test chamber are presented in Table 2. Water temperatures were within the $20 \pm 1^{\circ}\text{C}$ range established for the test. Dissolved oxygen concentrations remained ≥ 8.6 mg/L (95% of saturation) throughout the test. Measurements of pH ranged from 8.1 to 8.2 during the test. The measurements of hardness, alkalinity, specific conductance and TOC in the dilution water at test initiation were typical of Wildlife International, Ltd. well water (Table 3).

Daily observations of mortality and signs of toxicity observed during the test are presented in Table 4. There were two immobile daphnids in the negative control group at test termination. Current OECD and OPPTS guidelines state that up to 10% immobility/death is acceptable for control performance, therefore the two immobile daphnids (10%) in the negative control is acceptable. All other daphnids that were observed in the negative and solvent control groups appeared normal throughout the test. All daphnids that were observed in the 1.2 and 1.8 mg a.i./L treatment groups appeared normal throughout the test. Consequently, the no-mortality concentration and the NOEC were 1.8 mg a.i./L. EC50 values at 24 and 48 hours were estimated to be >1.8 mg a.i./L (Table 5).

CONCLUSIONS

The 48-hour EC50 value for the cladoceran, *Daphnia magna*, exposed to Tetrabromobisphenol A (TBBPA) under flow-through conditions was > 1.8 mg a.i./L, the highest test concentration tested. The NOEC and no mortality/immobility concentration was 1.8 mg a.i./L.

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REFERENCES

- 1 **Organisation for Economic Cooperation and Development.** 1984. OECD Guidelines for Testing of Chemicals. Guideline 202: *Daphnia sp. Acute Immobilization Test and Reproduction Test*. Updated Guideline, adopted April, 1984.
- 2 **U.S. Environmental Protection Agency.** 1996. OPPTS Number 850.1010: *Aquatic Invertebrate Acute Toxicity Test, Freshwater Daphnids*. Series 850 – Ecological Effects Test Guidelines (*draft*).
- 3 **ASTM Standard E729-88a.** 1994. *Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians*. American Society for Testing and Materials.
- 4 **APHA, AWWA, WPCF.** 1998. *Standard Methods for the Examination of Water and Wastewater*. 20th Edition, American Public Health Association. American Water Works Association. Water Pollution Control Federation, New York.

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Table 1

Measured Concentrations of Tetrabromobisphenol A (TBBPA) in Test Samples

Nominal Test Concentration (mg a.i./L)	Sample ID (439A-124-)	Sampling Time (Hours)	Measured Concentration (mg a.i./L) ^{1,2}	Percent of Nominal ²	Mean Measured Concentration (mg a.i./L)	Mean Percent of Nominal
Negative Control 0.0	1	0	< LOQ	--	--	--
	2	0	< LOQ	--		
	9	48	< LOQ	--		
	10	48	< LOQ	--		
Solvent Control 0.0	3	0	< LOQ	--	--	--
	4	0	< LOQ	--		
	11	48	< LOQ	--		
	12	48	< LOQ	--		
1.2	5	0	1.24	103	1.2	100
	6	0	1.25	104		
	13	48	1.18	98.7		
	14	48	1.19	99.3		
1.8	7	0	1.85	103	1.8	100
	8	0	1.82	101		
	15	48	1.74	96.7		
	16	48	1.71	95.3		

¹ The limit of quantitation (LOQ) was 0.250 mg a.i./L calculated as the product of the lowest calibration standard (0.0100 mg a.i./L) and the dilution factor of the matrix blanks (25).

² Results were generated using MacQuan version 1.6 software. Manual calculations may differ slightly.

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Table 2

Temperature, Dissolved Oxygen and pH of Water in the Test Chambers

Mean Measured Test Concentration (mg a.i./L)	Replicate	0 Hour			24 Hours		48 Hours		
		Temp. ¹ (°C)	DO ² (mg/L)	pH	DO (mg/L)	pH	Temp. (°C)	DO (mg/L)	pH
Negative Control	A	20.2	8.6	8.1	--	--	20.1	8.7	8.2
	B	20.2	--	--	8.7	8.1	20.1	--	--
Solvent Control	A	20.0	8.6	8.1	--	--	20.0	8.7	8.2
	B	20.0	--	--	8.7	8.1	20.0	--	--
1.2	A	20.1	8.7	8.1	--	--	20.0	8.8	8.2
	B	20.1	--	--	8.7	8.1	20.0	--	--
1.8	A	19.8	8.7	8.1	--	--	19.8	8.8	8.2
	B	19.8	--	--	8.7	8.2	19.8	--	--

¹ Manual temperature measurements. Temperature measured continuously during the test was approximately 20.0 to 20.5°C, measured to the nearest 0.5°C.

² A dissolved oxygen concentration of 6.8 mg/L represents 75% saturation at 20.0°C in freshwater.

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Table 3

Hardness, Alkalinity, Specific Conductance and Total Organic Carbon
Measured in Dilution Water at Test Initiation

Parameter	Day 0
Hardness (mg/L as CaCO ₃)	132
Alkalinity (mg/L as CaCO ₃)	185
Specific Conductance (µmhos/cm)	330
Total Organic Carbon (mg C/L)	<1

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Table 4

Cumulative Percent Mortality, Immobility and Treatment-Related Effects

Mean Measured Concentration (mg a.i./L)	Replicate	Daphnia/ Replicate	4.5 Hours ¹			24 Hours ¹			48 Hours			Percent Immobile and Dead
			Cumulative Dead	Number Immobile	Effects ²	Cumulative Dead	Number Immobile	Effects ²	Cumulative Dead	Number Immobile	Effects ²	
Negative Control	A	10	0	0	AN	0	0	AN	0	1	9 AN	10
	B	10	0	0	AN	0	0	AN	0	1	9 AN	
Solvent Control	A	10	0	0	AN	0	0	AN	0	0	10 AN	0
	B	10	0	0	AN	0	0	AN	0	0	10 AN	
1.2	A	10	0	0	AN	0	0	AN	0	0	10 AN	0
	B	10	0	0	AN	0	0	AN	0	0	10 AN	
1.8	A	10	0	0	AN	0	0	AN	0	0	10 AN	0
	B	10	0	0	AN	0	0	AN	0	0	10 AN	

¹ Daphnids were observed without removing the test compartments from the test solutions. Therefore, observations at 4.5 and 24-hours are estimates of mortality and immobility.² Observed Effects: AN = appear normal.

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Table 5

EC50 Values

Time	EC50 (mg a.i./L)	95% Confidence Interval (mg a.i./L)	Statistical Method
24 Hours	>1.8	-- ¹	NA ²
48 Hours	>1.8	-- ¹	NA ²

¹ 95% confidence limits could not be calculated from the data.² NA = not applicable; <50% mortality/immobility precluded statistical calculation of an EC50 value.

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Appendix 1

Specific Conductance, Hardness, Alkalinity and pH of Well Water Measured
During the 4-Week Period Immediately Preceding the Test

Parameter	Mean	Range
Specific Conductance ($\mu\text{mhos/cm}$)	313 (N = 4)	310 – 320
Hardness (mg/L as CaCO_3)	131 (N = 4)	124 – 140
Alkalinity (mg/L as CaCO_3)	184 (N = 4)	182 – 186
pH	8.1 (N = 4)	8.1 – 8.2

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Appendix 2

Analyses of Pesticides, Organics and Metals in Wildlife International, Ltd. Well Water ¹

Pesticides and Organics			
Component	Measured Concentration (ppb or ng/g)	Component	Measured Concentration (ppb or ng/g)
Aldicarb sulfone	< 50	Isofenphos	< 50
Aldicarb sulfoxide	< 50	Leptophos	< 50
Azinphos-ethyl	< 50	Linuron	< 50
Azinphos-methyl	< 50	Methidathion	< 50
Bifenox	< 50	Methiocarb	< 50
Bitertanol	< 50	Methomyl	< 50
Bromacil	< 50	Methoxychlor	< 250
Bromoxynil octanoic acid ester	< 50	Mirex	< 50
Captafol	< 50	Monocrotophos	< 50
Carbaryl	< 50	Myclobutanil	< 50
3-Hydroxy Carbofuran	< 50	Napropamide	< 50
Carbofuran	< 50	Norflurazon	< 50
Carbophenothion	< 50	Oxadiazon	< 50
cis-Chlordane	< 50	Oxamyl	< 50
trans-Chlordane	< 50	Oxyfluorfen	< 50
Chlorfenson	< 50	Paraoxon	< 50
trans-Chlorfenvinphos	< 50	cis-Permethrin	< 50
Chlorobenzilate	< 50	Perthane	< 50
Chloropropylate	< 50	Phosalone	< 50
Chloroxuron	< 50	Phosphamidon	< 50
Coumaphos	< 50	Piperalin	< 50
Crotoxypfos	< 50	Profenfos	< 50
Cyanazine	< 50	Promecarb	< 50
Cyfluthrin I	< 50	Propanil	< 50
Cypermethrin I	< 50	Propargite	< 50
o,p'-DDD	< 50	Propoxur	< 50
p,p'-DDE	< 50	Pyrethrin I	< 50
p,p'-DDD	< 50	Quinalphos	< 50
o,p'-DDT	< 50	Quinomethionate	< 50
p,p'-DDT	< 250	Quizalofop-ethyl	< 50
DEF	< 50	Sulprofos	< 50
Diclofop methyl	< 50	Tetrachlorovinphos	< 50
Dicrotophos	< 50	Tetradifon	< 50
Dieldrin	< 50	Thiobendazole	< 50
Diphenamid	< 50	Tilt I	< 50
Diuron	< 50	Tilt II	< 50
Endosulfan II	< 50	Trimethyl carbamate	< 50
Endrin	< 50		
Endrin ketone	< 50		
EPN	< 50		
Ethion	< 50		
Fenamiphos	< 50		
Fenarimol	< 50		
Fenobucarb	< 50		
Fenpropathrin	< 50		
Fensulfothion	< 50		
Fluzifop-P-butyl	< 50		

¹Analyses performed by Exygen Research on samples collected on July 31, 2002.

Appendix 2 (Continued)**Analyses of Pesticides, Organics and Metals in Wildlife International, Ltd. Well Water ¹**

Metals			
	(ppm or mg/L)		(ppm or mg/L)
Aluminum	< 0.204	Manganese	< 0.0153
Arsenic	< 0.0102	Mercury	< 0.0002
Beryllium	< 0.0051	Molybdenum	< 0.0005
Cadmium	< 0.0051	Nickel	< 5.1
Calcium	28.2	Potassium	5.45
Chromium	< 0.0102	Selenium	0.009
Cobalt	< 5.1	Silver	< 0.0102
Copper	< 0.0255	Sodium	18.6
Iron	< 5.1	Zinc	< 0.0204
Magnesium	11.6		

¹Analyses performed by Exygen Research on samples collected on July 31, 2002.

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Appendix 3

The Analysis of Tetrabromobisphenol A (TBBPA) in Freshwater

Appendix 3.1

Analytical Method Flowchart for the Analysis of TBBPA in Freshwater

**METHOD OUTLINE FOR THE ANALYSIS OF
TBBPA IN FRESHWATER**

Prepare quality control samples in freshwater using gas-tight syringes and volumetric flasks.
The matrix blank will be freshwater.



Dilute submitted samples and quality control samples with 50% (v/v) methanol NANOpure[®] water
solution using gas-tight syringes and volumetric flasks.



Transfer samples to autosampler vials and submit samples for
HPLC/MS analysis.

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Appendix 3.2

Typical HPLC/MS Operational Parameters

INSTRUMENT:	Hewlett-Packard Model 1100 High Performance Liquid Chromatograph with a Perkin-Elmer SCIEX API 100 Mass Spectrometer configured with a Heated Nebulizer ion source. Operated in negative selective ion monitoring mode.
ANALYTICAL COLUMN:	Keystone Betasil C ₁₈ column (50 mm × 2 mm, 3-μm particle size)
GUARD COLUMN:	Keystone Javelin C ₁₈ column (20 mm × 2 mm)
OVEN TEMPERATURE:	40°C
STOP TIME:	5.00 minutes
FLOW RATE:	0.250 mL/minute
MOBILE PHASE:	Solvent A: 0.1% formic acid in NANOpure [®] water (20%) Solvent B: Methanol (80%)
INJECTION VOLUME:	100 μL
TBBPA RETENTION TIME:	Approximately 3.6 minutes
TBBPA MONITORED MASS:	542.7 amu

Appendix 3.3**Analytical Stocks and Standards Preparation**

A primary stock solution of TBBPA was prepared by weighing 0.1008 g of the test substance on an analytical balance. The test substance (99.17% purity) was transferred to a 100-mL volumetric flask and brought to volume using methanol. The primary stock solution contained 1.00 mg a.i./mL of TBBPA. Secondary stocks (0.100, 0.0100, and 0.00100 mg a.i./mL TBBPA in methanol) were prepared from the primary stock by serial dilution. The primary and secondary stocks were used to fortify the quality control samples and to prepare calibration standards. Calibration standards for TBBPA were prepared in 50% (v/v) methanol in NANOpure[®] water solution. The following shows the dilution scheme for the calibration standards.

Stock Concentration (mg a.i./mL)	Aliquot (mL)	Final Volume (L)	Standard Concentration (mg a.i./L)
0.0100	0.100	0.100	0.0100
0.0100	0.250	0.100	0.0250
0.0100	0.500	0.100	0.0500
0.0100	0.750	0.100	0.0750
0.0100	1.00	0.100	0.100

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Appendix 3.4**Example Calculations for a Representative Sample**

The analytical result and percent recovery for sample number 439A-124-5, nominal concentration of 1.2 mg a.i./L, were calculated using the following equations:

$$\text{TBBPA (mg a.i./L) in sample} = \frac{\text{Peak area} - (\text{Y-intercept})}{\text{Slope}} \times \text{Dilution factor}$$

Peak Area = 606118

Y-intercept = 3517.6104

Slope = 12188727

Initial Volume (V_i): = 0.200 mL

Final Volume (V_f): = 5.00 mL

Dilution Factor (V_f/V_i): = 25

$$\text{TBBPA (mg a.i./L) in sample} = \frac{606118 - (3517.6104)}{12188727} \times 25$$

$$= 1.236 \text{ mg a.i./L}$$

$$\text{Percent of nominal concentration} = \frac{\text{TBBPA in sample (mg a.i./L)}}{\text{TBBPA nominal concentration (mg a.i./L)}} \times 100$$

$$= \frac{1.236}{1.2} \times 100$$

$$= 103\%$$

Results were generated using MacQuan version 1.6 software. Manual calculations may differ slightly.

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Appendix 3.5

Quality Control Samples of TBBPA in Freshwater

Sample Number (439A-123,124-)	Concentration (mg a.i./L)		Percent Recovery ²
	Fortified	Measured ^{1,2}	
MAB-2	0.0	< LOQ	--
MAB-3	0.0	< LOQ	--
MAS-4	1.00	0.988	98.8
MAS-5	1.50	1.49	99.2
MAS-6	2.00	2.02	101
MAS-7	1.00	1.01	101
MAS-8	1.50	1.50	100
MAS-9	2.00	2.05	103

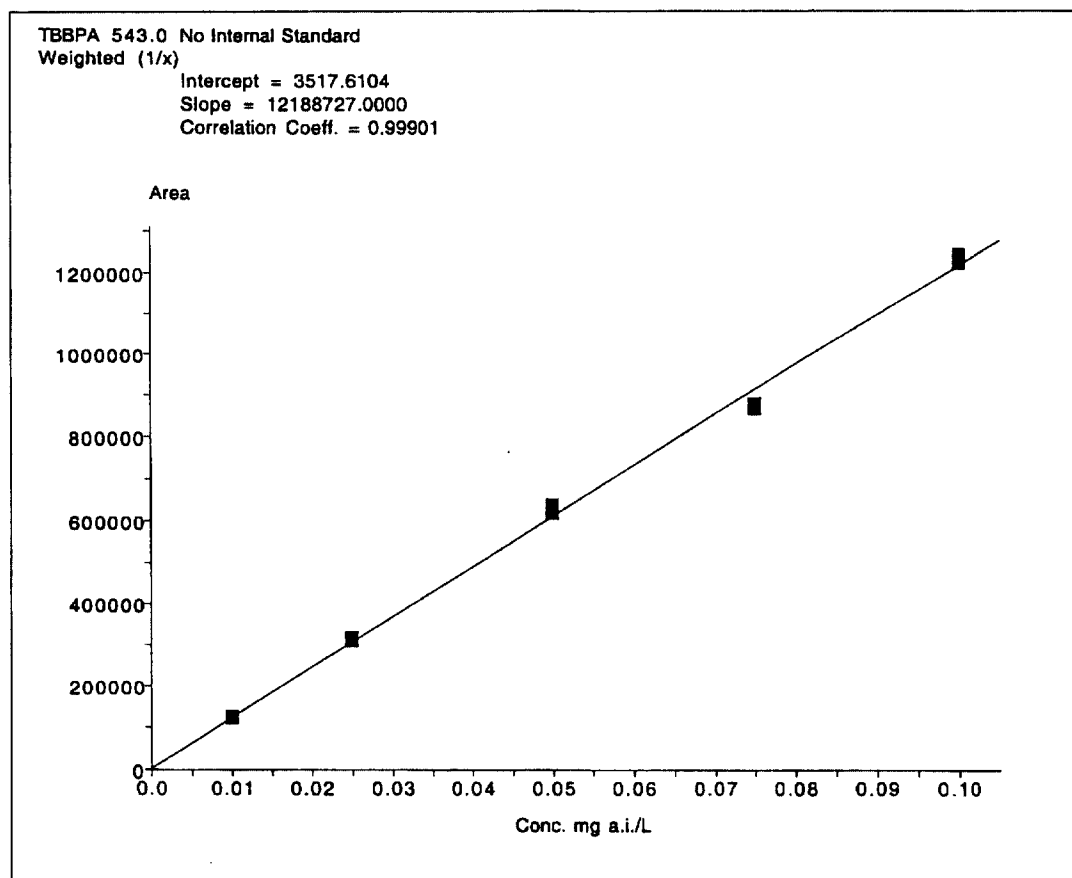
¹ The limit of quantitation (LOQ) was 0.250 mg a.i./L calculated as the product of the lowest calibration standard (0.0100 mg a.i./L) and the dilution factor of the matrix blanks (25).

² Results were generated using MacQuan version 1.6 software. Manual calculations may differ slightly.

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Appendix 3.6

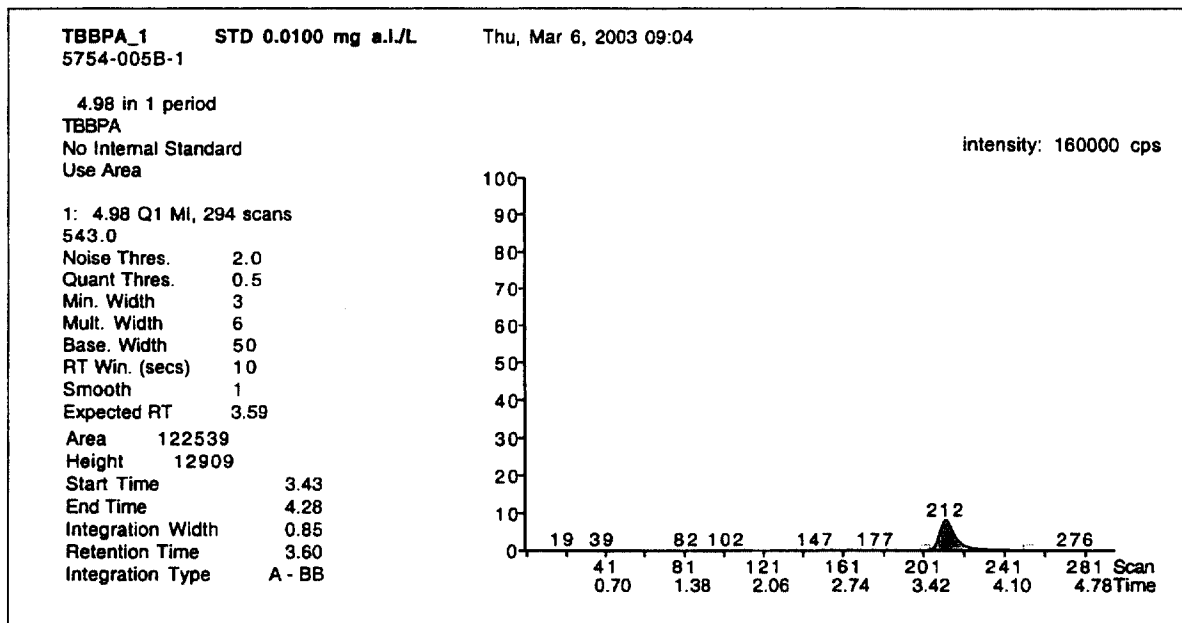
Representative Calibration Curve for TBBPA



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Appendix 3.7

Representative Chromatogram of a Low-level TBBPA Calibration Standard

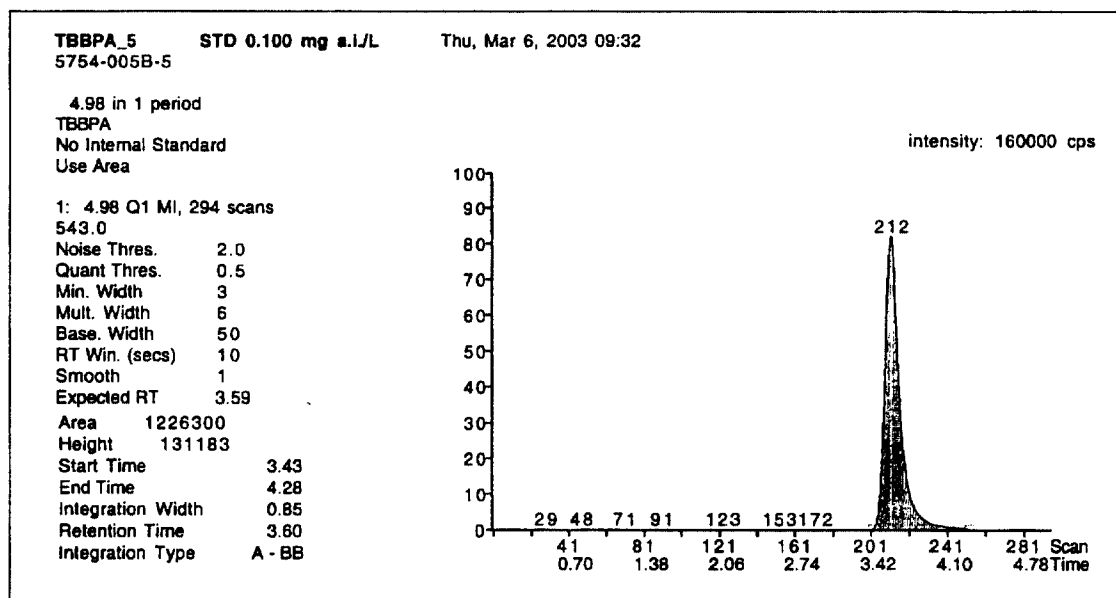


Nominal concentration: 0.0100 mg a.i./L

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Appendix 3.8

Representative Chromatogram of a High-level TBBPA Calibration Standard

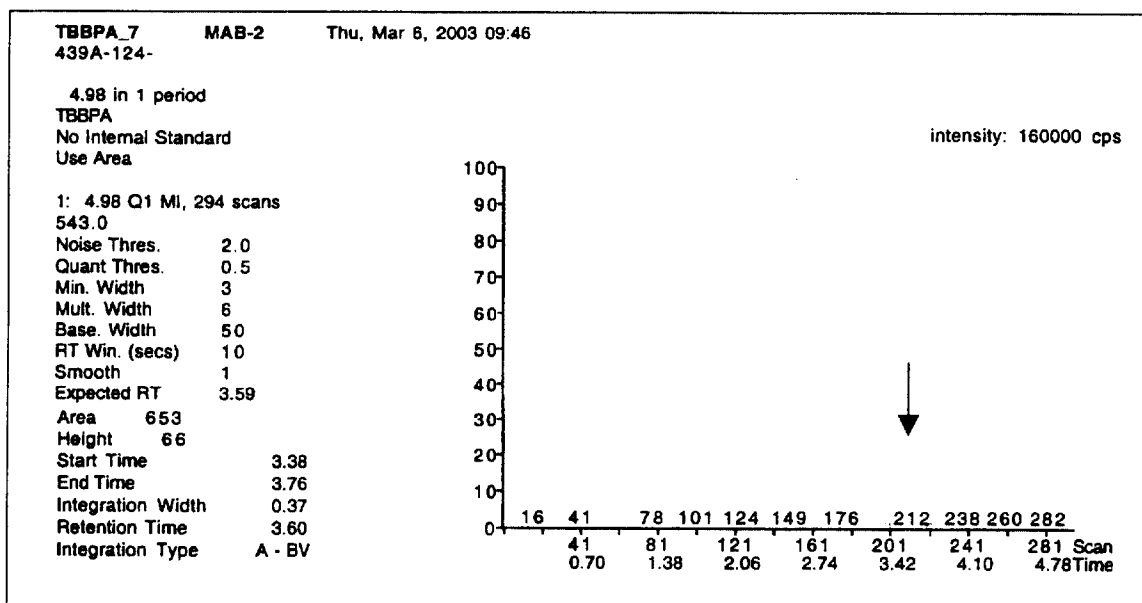


Nominal concentration: 0.100 mg a.i./L

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Appendix 3.9

Representative Chromatogram of a Matrix Blank Sample



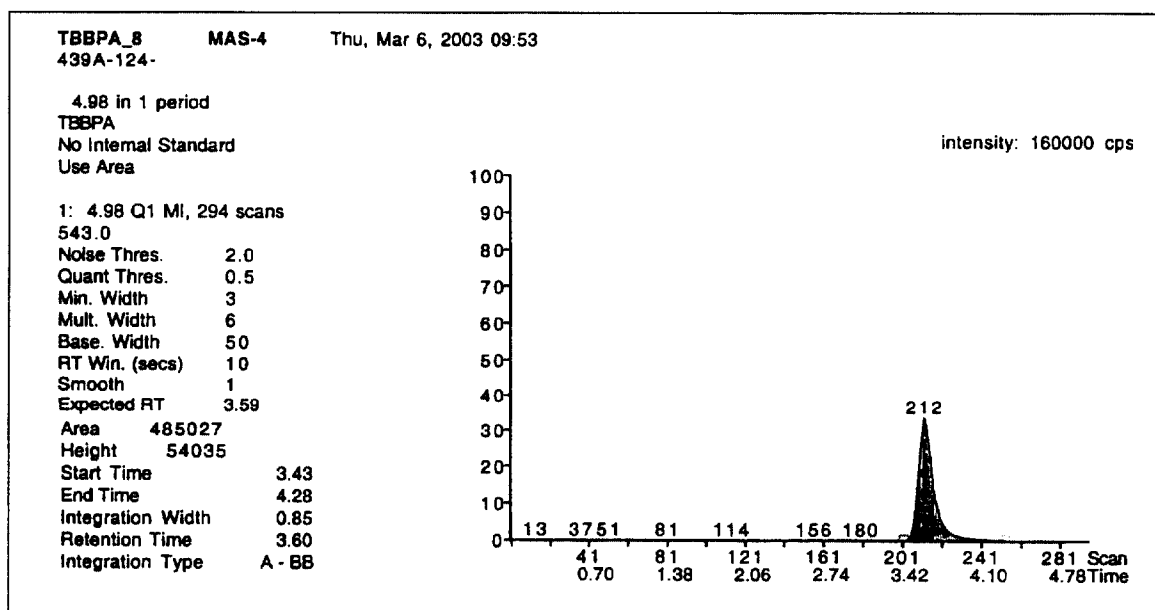
Sample number 439A-123,124-MAB-2. Dilution factor = 25X.

The arrow indicates the approximate retention time of TBBPA.

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Appendix 3.10

Representative Chromatogram of a Matrix Fortification Sample



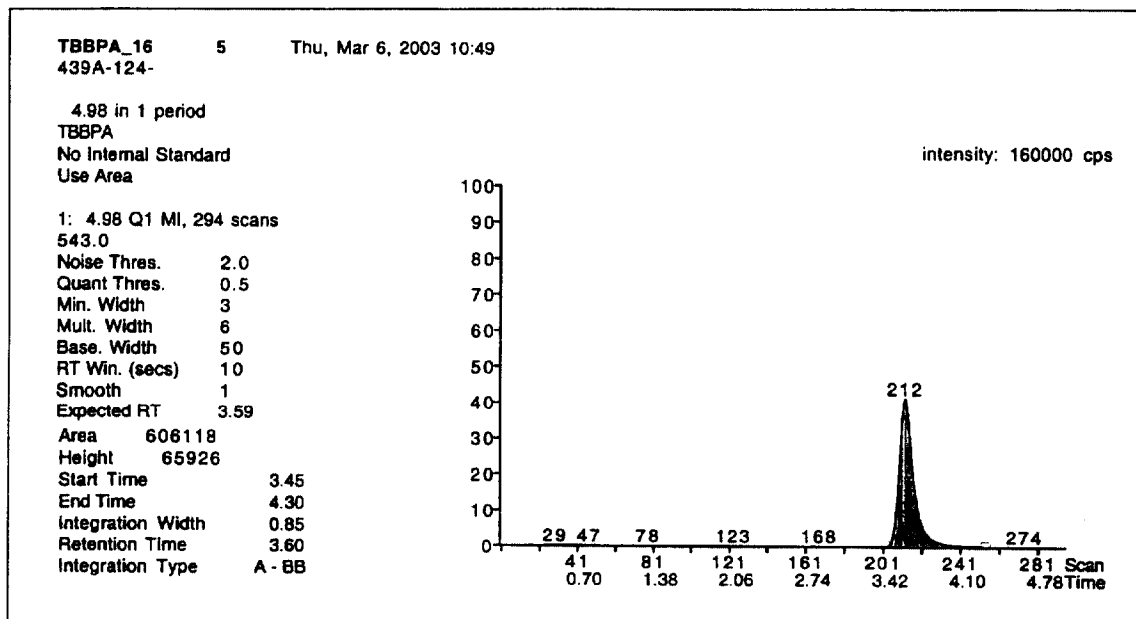
Sample number: 439A-123,124-MAS-4, nominal concentration 1.00 mg a.i./L.

Dilution factor = 25X.

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Appendix 3.11

Representative Chromatogram of a Test Sample



Sample number: 439A-124-5, Day 0, nominal concentration 1.2 mg a.i./L.
Dilution factor = 25X.

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Appendix 4

Protocol and Amendments

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PROTOCOL

TETRABROMOBISPHENOL A: A 48-HOUR FLOW-THROUGH ACUTE TOXICITY
TEST WITH THE CLADOCERAN (*Daphnia magna*)

OECD Guideline 202

U.S. EPA OPPTS Number 850.1010

Submitted to

American Chemistry Council's
Brominated Flame Retardant Industry Panel
1300 Wilson Boulevard
Arlington, Virginia 22209

Wildlife International, Ltd.

8598 Commerce Drive
Easton, Maryland 21601
(410) 822-8600

February 13, 2003

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Wildlife International, Ltd.

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**TETRABROMOBISPHENOL A: A 48-HOUR FLOW-THROUGH ACUTE TOXICITY
TEST WITH THE CLADOCERAN (*Daphnia magna*)**

SPONSOR: American Chemistry Council's
Brominated Flame Retardant Industry Panel
1300 Wilson Boulevard
Arlington, Virginia 22209

SPONSOR'S REPRESENTATIVE: Ms. Wendy Sherman

TESTING FACILITY: Wildlife International, Ltd.
8598 Commerce Drive
Easton, Maryland 21601

STUDY DIRECTOR: Amy Blankinship, Biologist
Wildlife International, Ltd.

LABORATORY MANAGEMENT: Henry O. Krueger, Ph.D.
Director of Aquatic Toxicology/Terrestrial Plants & Insects

FOR LABORATORY USE ONLY

Proposed Dates:	
Experimental Start Date: <u>3/5/03</u>	Experimental Termination Date: <u>3/7/03</u>
Project No.: <u>439A-124</u>	
Test Concentrations: <u>Negative Control, Solvent Control (0.1 mL DMF/L), 1.2 mg/L, 1.8 mg/L</u>	
Test Substance No.: <u>5754</u> Reference Substance No. (if applicable): <u> </u>	

PROTOCOL APPROVAL

<u>Amy Blankinship</u> STUDY DIRECTOR	<u>2/28/03</u> DATE
<u>H. O. Krueger</u> LABORATORY MANAGEMENT	<u>2/28/03</u> DATE
<u>Susan Hewitt for Wendy Sherman</u> SPONSOR'S REPRESENTATIVE	<u>2/26/03</u> DATE

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Wildlife International, Ltd.

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INTRODUCTION

Wildlife International, Ltd. will conduct a flow-through acute toxicity test with the cladoceran, *Daphnia magna*, for the Sponsor at the Wildlife International, Ltd. aquatic toxicology facility in Easton, Maryland. The study will be performed based on procedures in the OECD Guideline for Testing of Chemicals, 202: *Daphnia sp. Acute Immobilization Test and Reproduction Test* (1); U.S. EPA Series 850 - Ecological Effects Test Guidelines OPPTS Number 850.1010: *Aquatic Invertebrate Acute Toxicity Test* (2); and ASTM Standard E-729-88a: *Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates and Amphibians* (3). Raw data for all work performed at Wildlife International, Ltd. and a copy of the final report will be filed by project number in archives located on the Wildlife International, Ltd. site, or at an alternative location to be specified in the final report.

OBJECTIVE

The objective of this study is to determine the acute effects of Tetrabromobisphenol A (TBBPA) on the cladoceran, *Daphnia magna*, under flow-through test conditions for a period of 48 hours.

EXPERIMENTAL DESIGN

Daphnids will be exposed to a two test concentrations (1.2 and 1.8 ppm), a negative (dilution water) control and a solvent control for 48 hours. Two replicate test chambers will be maintained in each treatment and control group, with 10 neonate daphnids in each chamber so that a total of 20 neonate daphnids are exposed in each treatment and control group.

Nominal test concentrations were selected in consultation with the Sponsor based upon information such as known toxicity data, physical/chemical properties of the test substance or other relevant information. Water samples will be collected from appropriate test chambers at specified intervals for analysis of the test substance. Results of analyses will be used to calculate mean measured test concentrations.

To control bias, neonate daphnids will be impartially assigned to transfer chambers at test initiation. No other potential sources of bias are expected to affect the results of the study. EC50 values will be calculated, when possible, based on the number of dead or immobilized daphnids observed in each test concentration after each 24-hour interval of exposure.

*Wildlife International, Ltd.***MATERIALS AND METHODS****Test Substance**

The test substance consisted of a composite of TBBPA samples received from three manufacturers. The material's identity and date received from each of the manufacturers is given below:

<u>Manufacturer</u>	<u>Lot/Batch</u>	<u>Date Received</u>	<u>Wildlife International Ltd. Identification Number</u>
Great Lakes Chemical Corporation	1008JE04B	August 16, 2001	5722
Albemarle Corporation	25115T-1	August 16, 2001	5721
Bromine Compounds, Ltd.	010040	August 31, 2001	5733

The composite test substance was assigned Wildlife International Ltd. identification number 5754 and was stored under ambient conditions. Subsamples of the composite test substance were shipped to Albemarle Corporation for characterization and purity analyses. The results of the analyses indicated the composite test substance was homogeneous and contained TBBPA with a purity of 99.17%. The test substance was stored at room temperature.

The Sponsor is responsible for all information related to the test substance and agrees to accept any unused test substance and/or test substance containers remaining at the end of the study.

Preparation of Test Concentrations

The test substance will be administered to the test organism in water. This route of administration was selected because it represents the most likely route of exposure to aquatic organisms.

The test substance will be mixed directly with dilution water or may be first mixed with a solvent. If a solvent is used, the test substance will be dissolved in the solvent to form a stock solution that will subsequently be added to the dilution water. Reverse osmosis water will be the solvent of choice, although dimethyl formamide, triethylene glycol, methanol, ethanol, or acetone may be used. If an organic solvent is required, a solvent control will be included in the experimental design along with a negative (dilution water) control group. The concentration of the organic solvent will not exceed 0.1-mL/L, when possible. The solvent concentration in the solvent control will be equal to the highest solvent concentration in test chambers containing the test substance.

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Test Organism

The cladoceran, *Daphnia magna*, has been selected as the test species for this study. Daphnids are representative of an important group of aquatic invertebrates, and have been selected for use in the test based upon past use history and ease of culturing in the laboratory. Daphnid neonates to be used in the test will be less than 24 hours old and will be obtained from cultures maintained at Wildlife International, Ltd., Easton, Maryland. The identity of the species will be verified by the supplier of the original culture or by Wildlife International, Ltd. using appropriate taxonomic keys such as Pennak (4).

Daphnids will be cultured in water from the same source and at approximately the same temperature as will be used during the test. Daphnids in the cultures producing neonates for the test will be held for at least 10 days prior to collection of the neonates for testing. Adult daphnids in the culture will produce an average of at least 3 young per adult per day over the 7 day period prior to the test. Neonates from daphnids that show signs of disease or stress will not be used as test organisms. Daphnids in holding that produce ephippia also will not be used to supply neonates for testing.

Daphnids in the cultures will be fed at least once daily. The diet will be a mixture of yeast, Cerophyll®, and trout chow (YCT), supplemented with a suspension of the freshwater green alga *Selenastrum capricornutum*. Adults are fed during the 24-hour period prior to test initiation, but neonates will not be fed during the test. Specifications for acceptable levels of contaminants in daphnid diets have not been established. However, there are no known levels of contaminants reasonably expected to be present in the diet that are considered to interfere with the purpose or conduct of the test.

Neonates will be obtained for testing from at least three individual adults that have produced at least one previous brood. Prior to test initiation, the neonates will be collected from cultures and transferred to small containers. The daphnids will be released into the test compartments below the water surface using a wide-bore pipette.

Dilution Water

The water used for culturing and testing will be obtained from a well approximately 40 meters deep located on the Wildlife International, Ltd. site. The water will be passed through a sand filter and pumped into a 37,800-L storage tank where the water will be aerated with spray nozzles. Prior to use the water will be filtered to 0.45 µm in order to remove fine particles, and may be UV-sterilized. Water used

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for culturing and testing is characterized as moderately hard. Typical values for hardness, alkalinity, pH and specific conductance are approximately:

Hardness, mg/L as CaCO ₃	145
Alkalinity, mg/L as CaCO ₃	190
pH	8.1
Specific Conductance, μ mhos/cm	330

Hardness, alkalinity, pH and specific conductance will be measured weekly to monitor the consistency of the well water. Means and ranges of the measured parameters for the four-week period preceding the test will be provided in the final report. Analyses will be performed at least once annually to determine the concentrations of selected organic and inorganic constituents in the water and results of the most recent GLP compliant analyses will be summarized in the final report.

Test Apparatus

A continuous-flow diluter will be used to provide each concentration of the test substance, a negative (dilution water) control, and a solvent control, when necessary. A syringe pump, peristaltic pump, or a similar device will be used to deliver the test substance to mixing chambers where the test substance will be mixed with dilution water. The flow of dilution water into each mixing chamber will be controlled using rotameters. After mixing, test solutions will be split to each replicate chamber. The proportion of water split to each replicate will be checked prior to the test to ensure that these flow rates vary by no more than $\pm 10\%$ of the mean of the two replicates.

In tests where solvent controls are required, the solvent will be injected into a mixing chamber where it will be diluted to the appropriate concentration with dilution water. The concentration of solvent in the solvent control will be equal to that in the highest treatment level.

The diluter will be adjusted so that each test chamber receives at least 5 volume additions of test solution every 24 hours. Test substance stock delivery pumps and rotameters will be calibrated before each test, and the delivery of test substance to test chambers will begin at least 4 hours prior to the test in order to establish equilibrium concentrations of the test substance. The general operation of the diluter will be checked visually at least two times per day during the test and at least once at the beginning and end of the test.

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Test compartments will be constructed from glass beakers 6.5 cm in diameter and 12 cm in height. Nylon screens will be attached to two holes in the side of each glass beaker. The beakers will be suspended in 8-L, Teflon®-lined polyethylene or stainless steel chambers filled with approximately 6.5 L of test solution. Test chambers will be indiscriminately positioned in a temperature-controlled water bath to maintain a temperature of $20 \pm 1^\circ\text{C}$. Test chambers will be labelled with project number, test concentration and replicate.

Environmental Conditions

Lighting used to illuminate the cultures and test chambers during culturing and testing will be provided by fluorescent tubes that emit wavelengths similar to natural sunlight (e.g., Colortone® 50). A photoperiod of 16 hours of light and 8 hours of dark will be controlled with an automatic timer. A 30-minute transition of low light intensity will be provided when lights go on and off to avoid sudden changes in light intensity. Light intensity will be measured at test initiation with a SPER Scientific Ltd. light meter or equivalent.

The target test temperature will be $20 \pm 1^\circ\text{C}$. Temperature will be measured in each test chamber at the beginning and end of the test using a liquid-in-glass thermometer. Temperature also will be measured with a continuous recorder in one negative control chamber. Recorder measurements will be verified with a liquid-in-glass thermometer prior to test initiation.

Dissolved oxygen and pH will be measured in alternate replicates of each treatment and control group at test initiation and at approximately 24-hour intervals during the test. In the event that dissolved oxygen concentrations fall below 75% of saturation, dissolved oxygen measurements will be taken in every test chamber and appropriate actions will be taken after consultation with the Sponsor. Dissolved oxygen concentrations will be measured with a Thermo Orion Model 850Aplus dissolved oxygen meter, or equivalent and pH will be measured with a Thermo Orion Model 720Aplus pH meter, or equivalent. If a treatment replicate reaches 100% mortality, dissolved oxygen, pH and temperature measurements will be taken in that replicate at that time, and then discontinued.

Hardness, alkalinity, specific conductance and total organic carbon (TOC) will be measured in the dilution water at the beginning of the test. Hardness and alkalinity measurements will be made by titration using procedures based on methods in *Standard Methods for the Examination of Water and*

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Wastewater (5). Specific conductance will be measured using a Yellow Springs Instrument Model 33 Salinity-Conductivity-Temperature meter, or equivalent. Total organic carbon will be measured on a Shimadzu Model 5000 TOC analyzer, or equivalent. Additional water quality measurements may be taken as deemed necessary by study personnel. The reason for the additional measurements will be documented in the raw data and summarized in the final report.

Biological Measurements

Observations of mortality, immobilization and clinical signs of toxicity will be made between 0-24 hours, and at 24 and 48 hours \pm 1 hour. Immobilization is defined as a lack of movement by the test organism except for minor activity of the appendages.

Sampling for Analytical Measurements

Water samples will be collected from each test chambers at the beginning and at the end of the test to determine concentrations of the test substance. In the event that 100% mortality occurs in any treatment, then sampling of that treatment will terminate following the next sampling interval. Samples will be collected at mid-depth from each test chamber and analyzed immediately, or placed in an appropriate storage container (e.g., glass or polypropylene bottle) and stored under refrigeration until analyzed. The sample scheme is summarized below:

PROPOSED NUMBERS OF VERIFICATION SAMPLES

Experimental Group	0 Hours	48 Hours
Control	2	2
Solvent Control (if needed)	2	2
Level 1- 1.2 ppm	2	2
Level 2- 1.8 ppm	2	2
	8	8

Total Number of Verification Samples = 16

The above numbers of samples represent those collected from the test and do not include quality control (QC) samples such as matrix blanks and fortifications prepared and analyzed during the analytical chemistry phase of the study. At the discretion of the Study Director, water samples from one or more appropriate test chambers and/or stock solutions will be collected and analyzed if an analytical error in

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sampling or analysis is suspected, or if a malfunction in the test substance delivery system occurs. The reason for the additional samples will be described by the Study Director and documented in the raw data and final report.

Analytical Measurements

Chemical analysis of the samples will be performed by Wildlife International, Ltd. The analytical method used will be based upon chromatographic methodology provided by the Sponsor and/or developed at Wildlife International, Ltd. The methodology used to analyze the test samples will be documented in the raw data and summarized in the final report.

Data Analysis

When the dose-response pattern allows calculation of an EC50 value, the data will be analyzed using the computer software of C.E. Stephan (6). The program was designed to calculate the EC50 value and the 95% confidence interval by probit analysis, the moving average method, or binomial probability with nonlinear interpolation (7,8,9). The EC50 value will be calculated, when possible, using mortality/immobility data collected at 24 and 48 hours. Additional analysis of data may be conducted if deemed appropriate by the Study Director. The results of the analysis will be documented in the raw data and summarized in the final report.

RECORDS TO BE MAINTAINED

Records to be maintained for data generated by Wildlife International, Ltd. will include, but not be limited to:

1. A copy of the signed protocol.
2. Identification and characterization of the test substance, if provided by the Sponsor.
3. Dates of initiation and termination of the test.
4. Daphnid history and culture records.
5. Results of rangefinding tests, when applicable.
6. Stock solution calculation and preparation.
7. Daily observations.
8. Water chemistry results (e.g., hardness and alkalinity).
9. If applicable, the methods used to analyze test substance concentrations and the results of analytical measurements.

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10. Statistical calculations.
11. Test conditions (light intensity, photoperiod, etc.).
12. Calculation and preparation of test concentrations.
13. Copy of final report.

FINAL REPORT

A final report of the results of the study will be prepared by Wildlife International, Ltd. The report will include the following, when applicable:

1. Name and address of the facility performing the study.
2. Dates upon which the study was initiated and completed, and the definitive experimental start and termination dates.
3. A statement of compliance signed by the Study Director addressing any exceptions to Good Laboratory Practice Standards.
4. Objectives and procedures, as stated in the approved protocol, including all changes to the protocol.
5. The test substance identification including name, chemical abstract number or code number, strength, purity, composition, and other information provided by the Sponsor.
6. Stability and solubility of the test substance under the conditions of administration, if provided by the Sponsor.
7. A description of the methods used to conduct the test.
8. A description of the test organisms, including the source, scientific name, age or life stage and feed types.
9. A description of the preparation of the test solutions.
10. The methods used to allocate organisms to test chambers and begin the test, the number of organisms and chambers per treatment, and the duration of the test.
11. A description of circumstances that may have affected the quality or integrity of the data.
12. The name of the Study Director and the names of other scientists, professionals, and supervisory personnel involved in the study.
13. A description of the transformations, calculations, and operations performed on the data, a summary and analysis of the biological data and analytical chemistry data, and a statement of the conclusions drawn from the analyses. A graph plotting the concentration response curve at 48

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Wildlife International, Ltd.

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- hours, when sufficient data exists. If the data is conducive to evaluation by probit analysis, the slope of the concentration-response curve will be reported.
14. Statistical methods used to evaluate the data.
 15. The signed and dated reports of each of the individual scientists or other professionals involved in the study, if applicable.
 16. The location where raw data and final report are to be stored.
 17. A statement prepared by the Quality Assurance Unit listing the dates that study inspections and audits were made and the dates of any findings reported to the Study Director and Management.
 18. If it is necessary to make corrections or additions to a final report after it has been accepted, such changes will be made in the form of an amendment issued by the Study Director. The amendment will clearly identify the part of the final report that is being amended and the reasons for the amendment, and will be signed by the Study Director.

CHANGES TO PROTOCOL

Planned changes to the protocol will be in the form of written amendments signed by the Study Director and approved by the Sponsor's Representative. Amendments will be considered as part of the protocol and will be attached to the final protocol. Any other changes will be in the form of written deviations signed by the Study Director and filed with the raw data. All changes to the protocol will be indicated in the final report.

GOOD LABORATORY PRACTICES

This study will be conducted in accordance with Good Laboratory Practice Standards for EPA (40 CFR Part 160 and/or Part 792); OECD Principles of Good Laboratory Practices (ENV/MC/CHEM (98) 17); and Japan MAFF (11 NohSan, Notification No. 6283, Agricultural Production Bureau, 1 October 1999). Each study conducted by Wildlife International, Ltd. is routinely examined by the Wildlife International, Ltd. Quality Assurance Unit for compliance with Good Laboratory Practices, Standard Operating Procedures and the specified protocol. A statement of compliance with Good Laboratory Practices will be prepared for all portions of the study conducted by Wildlife International, Ltd. The Sponsor will be responsible for compliance with Good Laboratory Practices for procedures performed by other laboratories (e.g., residue analyses or pathology). Raw data for all work performed at Wildlife International, Ltd. and a copy of the final report will be filed by project number in archives located on the Wildlife International, Ltd. site or at an alternative location to be specified in the final report.

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REFERENCES

- 1 **Organization for Economic Cooperation and Development.** 1984. Guideline 202: *Daphnia sp. Acute Immobilization Test and Reproduction Test*. OECD Guideline for Testing of Chemicals. Updated Guideline, adopted April, 1984.
- 2 **U.S. Environmental Protection Agency.** 1996. *Aquatic Invertebrate Acute Toxicity Test, Freshwater Daphnids*. Series 850-Ecological Effects Test Guidelines (*draft*), OPPTS Number 850.1010.
- 3 **ASTM Standard E729-88a.** 1994. *Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians*. American Society for Testing and Materials.
- 4 **Pennak, R.W.** 1978. *Freshwater Invertebrates of the United States*. 2nd Ed. 365 p.
- 5 **APHA, AWWA, WPCF.** 1998. *Standard Methods for the Examination of Water and Wastewater*. 20th Edition, American Public Health Association. American Water Works Association. Water Pollution Control Federation, New York.
- 6 **Stephan, C.E.** 1978. U.S. EPA, Environmental Research Laboratory, Duluth, Minnesota. Personal communication.
- 7 **Finney, D.J.** 1971. *Statistical Methods in Biological Assay*. Second edition. Griffin Press, London.
- 8 **Thompson, W.R.** 1974. *Bacteriological Reviews*. Vol. II, No. 2. Pp. 115-145.
- 9 **Stephan, C.E.** 1977. Methods for Calculating an LC50, *Aquatic Toxicology and Hazard Evaluations*. American Society for Testing and Materials. Publication Number STP 634. Pp 65-84.

Wildlife International, Ltd.

Project Number 439A-124
Page 1 of 2

AMENDMENT TO STUDY PROTOCOL

STUDY TITLE: Tetrabromobisphenol A: A 48-Hour Flow-Through Acute Toxicity Test with the Cladoceran (*Daphnia magna*)

PROTOCOL NO.: 439/021303/DAP-48H2/OECD-OPPTS/SUB439 **AMENDMENT NO.:** 1

SPONSOR: American Chemistry Council's Brominated Flame Retardant Industry Panel **PROJECT NO.:** 439A-124

EFFECTIVE DATE: March 3, 2003

AMENDMENT: Test Apparatus, Pages 6 and 7:

CHANGE: Test compartments will be constructed from glass beakers 6.5 cm in diameter and 12 cm in height. Nylon screens will be attached to two holes in the side of each glass beaker. The beakers will be suspended in 8-L, Teflon®-lined polyethylene or stainless steel chambers filled with approximately 6.5L of test solution. Test chambers will be indiscriminately positioned in a temperature-controlled water bath to maintain a temperature of $20 \pm 1^{\circ}\text{C}$.

TO: Test compartments will be constructed from glass cylinders approximately 50 mm in diameter, with 425- μm nylon or Teflon® screen attached to the bottom using silicone sealant. The cylinders will be inverted inside 150-mL glass beakers. The beakers will be submerged in 9-L glass aquaria filled with approximately 7 L of test solution. Test chambers will be indiscriminately positioned in a temperature-controlled environmental chamber set to maintain a temperature of $20 \pm 1^{\circ}\text{C}$.

REASON: This change is in an effort to help prevent the Daphnids from becoming trapped on air bubbles that form on the test chambers and compartments in the test apparatus.

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Wildlife International, Ltd.

Project Number 439A-124
Page 2 of 2

AMENDMENT: Test Organism, Page 5:

CHANGE: Prior to test initiation, the neonates will be collected from cultures and transferred to small containers. The daphnids will be released into the test compartments below the water surface using a wide-bore pipette.

TO: Prior to test initiation, the neonates will be collected from cultures and impartially transferred 1 or 2 at a time to small glass containers until each contains 10 neonates. Each transfer container will be impartially assigned to a test chamber, and placed inside the test compartment. The neonates will be released into the test compartment by gradually submerging the test compartments in the test chamber. The test compartments and transfer containers will remain submerged in the test chamber throughout the exposure period.

REASON: To clarify how the organisms will be distributed to the test chambers so that the neonates will not become trapped in the air/water interface during the test.

AMENDMENT: Biological Measurements, Page 8:

ADD: Observations for the 0-24 and 24-hour intervals will be conducted without removing the test compartments from the test solution. Therefore, these observations will be estimates, rather than exact counts. An exact count of mortalities, immobile organisms and effects will be made at test termination.

REASON: Numbers will be estimated in order to minimize disturbance of the test organisms in the compartments during the test.

Amy Blankinship
STUDY DIRECTOR

3-5-03 ^{slightly past 3:00}
DATE

[Signature]
LABORATORY MANAGEMENT

3/10/03
DATE

Wildlife International, Ltd.

Project Number 439A-124
Page 1 of 1

AMENDMENT TO STUDY PROTOCOL

STUDY TITLE: Tetrabromobisphenol A: A 48-Hour Flow-Through Acute Toxicity Test with the Cladoceran (*Daphnia magna*)

PROTOCOL NO.: 439/021303/DAP-48H2/OECD-OPPTS/SUB439 **AMENDMENT NO.:** 2

SPONSOR: American Chemistry Council's Brominated Flame Retardant Industry Panel **PROJECT NO.:** 439A-124

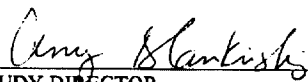
EFFECTIVE DATE: March 6, 2003

AMENDMENT: References, Page 12:

CHANGE: Thompson, W.R. 1974. *Bacteriological Reviews*. Vol. II, No. 2. Pp. 115-145.

TO: Thompson, W.R. 1947. *Bacteriological Reviews*. Vol. II, No. 2. Pp. 115-145.

REASON: Year is incorrect.



STUDY DIRECTOR

3-10-03
DATE



LABORATORY MANAGEMENT

3-12-03
DATE

QA Reviewed by JEB 3.7.03

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Wildlife International, Ltd.

Project Number 439A-124
Page 1 of 1

AMENDMENT TO STUDY PROTOCOL

STUDY TITLE: Tetrabromobisphenol A: A 48-Hour Flow-Through Acute Toxicity Test with the Cladoceran (*Daphnia magna*)

PROTOCOL NO.: 439/021303/DAP-48H2/OECD-OPPTS/SUB439 **AMENDMENT NO.:** 3

SPONSOR: American Chemistry Council's Brominated Flame Retardant Industry Panel **PROJECT NO.:** 439A-124

EFFECTIVE DATE: July 3, 2003

AMENDMENT: Objective, Page 3:

CHANGE: The objective of this study is to determine the acute effects of Tetrabromobisphenol A (TBBPA) on the cladoceran, *Daphnia magna*, under flow-through test conditions for a period of 48 hours.

TO: The objective of this study is to determine if the acute toxicity of Tetrabromobisphenol A (TBBPA) to the cladoceran, *Daphnia magna*, during a 48-hour exposure period under flow-through test conditions would be > 1.0 mg active ingredient (a.i.)/L.

REASON: To add additional wording requested by the sponsor.



STUDY DIRECTOR

7-8-03

DATE



LABORATORY MANAGEMENT

7-8-03

DATE

Reviewed by QA (SLC) 7-3-03

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Appendix 5

Analytical Report for Tetrabromobisphenol A (TBBPA)

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**ALBEMARLE CORPORATION
RESEARCH AND DEVELOPMENT DEPARTMENT**

**FINAL REPORT ON THE CHEMICAL CHARACTERIZATION (IDENTITY AND PURITY)
OF TETRABROMOBISPHENOL-A (TBBPA) IN SUPPORT OF "A 48-HOUR FLOW-
THROUGH ACUTE TOXICITY TEST WITH THE CLADOCERAN (*Daphnia magna*) AND A
96-HOUR FLOW-THROUGH ACUTE TOXICITY TEST WITH RAINBOW TROUT
(*Oncorhynchus mykiss*)", CONDUCTED BY WILDLIFE INTERNATIONAL, LTD.**

- I. Protocol Number: TBBPA-05-29-2003
- II. Sponsor: American Chemistry Council
Brominated Flame Retardant Industry Panel
1300 Wilson Boulevard
Arlington, Virginia 22209
Study Monitor: Wendy K. Sherman
- III. Analytical Testing Facilities: Albemarle Corporation
Process Development Center
Gulf States Road
Baton Rouge, LA 70805
Study Chemist: Paul F. Ranken, Ph. D.
- IV. Date of Study Initiation: June 2, 2003
Date of Study Completion: June 16, 2003
- V. Test Article: A sample of the test article, an end of study sample of Tetrabromobisphenol-A (WIL Test Substance 5754), was analyzed at the Albemarle Process Development Center. WIL Test Substance 5754 is a composite of commercial product from Albemarle Corporation, Great Lakes Chemical Corporation and the Dead Sea Bromine Group. Wildlife International Ltd., Easton, MD 21601, prepared the composite.
- VI. Objective/Methodology: This study was initiated to confirm the identity of the test article and to demonstrate the purity of the test article. Fourier Transform Infrared Spectroscopy using SOP No. ARS-284-R4 confirmed the identity of the test article. In this procedure, the test article infrared spectrum was compared to a standard reference spectrum of TBBPA (Aldrich TBBPA, lot 03120DI). High Performance Liquid Chromatography (HPLC)

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using SOP No. ARS-443-R2 determined the purity (area % TBBPA) of the test article. The test article was further characterized by measuring the concentration (area%) of three potential impurities: tribromophenol, tribromobisphenol-A and o,p'-tetrabromobisphenol-A. Chain of Custody and sample handling were conducted according to established standard operating procedures.

VII. Results:

The Table contains the test article analytical data from the study. Fourier Transform Infrared Spectroscopy confirmed the identity of the test article. High Performance Liquid Chromatography (HPLC) determined the purity of the test article to be 99.27%. Further characterization of the test article was accomplished by measuring the concentration of the three expected impurities. There were no circumstances that may have affected the quality or integrity of the data.

VIII. Regulatory Requirements:

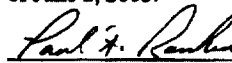
The study conformed to the requirements of EPA TSCA (40 CFR Part 792) Good Laboratory Practice Regulations and the OECD [C(97)186/Final] Good Laboratory Practice Regulations.

IX. Data/Record Retention:

All log books, spectra and reports will be forwarded to the Quality Assurance Unit (QAU) for a final review prior to filing in the designated Health and Environment archives at Albemarle Corporation, Health and Environment Department, 451 Florida Street, Baton Rouge, LA 70801.

X. Protocol Amendment:

The protocol was amended on June 12, 2003 to reflect a change in the approval date. The approval date originally listed by the study chemist was incorrectly listed as June 30, 2003. The amendment shows the correct approval date of June 2, 2003.


Paul F. Ranken, Ph.D.
STUDY CHEMIST

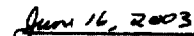
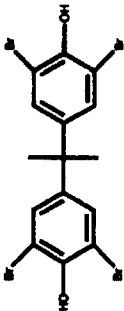

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Table 1. CONCLUSIONS AND TEST ARTICLE ANALYTICAL DATA FOR TBBPA, WIL Test Substance #5754

CHEMICAL NAME: Tetrabromobisphenol-A			
C.A.S. No.: 79-94-7			
MOLECULAR FORMULA: $C_{15}H_{12}Br_4O_2$			
PHYSICAL FORM: White Powder			
CHEMICAL STRUCTURE:			
			
ANALYSIS	RESULTS		ANALYST
	Area %		
	TBBPA	2,4,6-Tribromophenol	0,p-tetrabromobisphenol-A
HPLC	99.27	0.01	0.68
FT-IR	The sample FT-IR spectrum matched that of the Aldrich reference spectrum. All spectra are on file with the original data.		
CONCLUSION:	Based on these analytical data, the test article identity was confirmed as tetrabromobisphenol-A. The test article was shown to have a purity of 99.27%.		
		06/07/03	J.S. Arroyave
		06/06/03	W.T. Cobb

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**CHEMICAL CHARACTERIZATION ANALYTICAL PROTOCOL
FOR DETERMINING THE IDENTITY AND PURITY
OF TETRABROMOBISPHENOL-A (TBBPA) IN SUPPORT OF "A 48-HOUR
FLOW-THROUGH ACUTE TOXICITY TEST WITH THE CLADOCERAN (*Daphnia magna*) AND A 96-HOUR FLOW-THROUGH ACUTE TOXICITY TEST WITH
RAINBOW TROUT (*Oncorhynchus mykiss*)", CONDUCTED BY WILDLIFE
INTERNATIONAL, LTD.**

PROTOCOL AMENDMENT

Date: June 16, 2003

Section to be changed: Section X, page 2

Change: Date of protocol approval is June 2, 2003.

Reason for change: The protocol approval date originally listed by the Study Chemist was incorrectly given as June 30, 2003. The amendment shows the correct approval date of June 2, 2003.

June 16, 2003
Date:

Paul F. Ranken
Paul F. Ranken
Study Chemist

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Appendix 6

Personnel Involved in the Study

The following key Wildlife International, Ltd. personnel were involved in the conduct or management of this study:

1. Henry O. Krueger, Ph.D., Director of Aquatic Toxicology/Terrestrial Plants and Insects
2. Willard B. Nixon, Ph.D., Director of Chemistry
3. Cary A. Sutherland, Laboratory Supervisor
4. Raymond L. Van Hoven, Scientist
5. Frank J. Lezotte, Chemist
6. Amy S. Blankinship, Aquatic Biologist

TETRABROMOBISPHENOL A:
A 96-HOUR FLOW-THROUGH ACUTE TOXICITY TEST
WITH THE RAINBOW TROUT (*Oncorhynchus mykiss*)

FINAL REPORT

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER : 439A-123

OECD GUIDELINE 203

and

U.S. EPA OPPTS 850.1075

AUTHORS :

Amy S. Blankinship, M.S.
Raymond L. Van Hoven, Ph.D.
Henry O. Krueger, Ph.D.

STUDY INITIATION DATE: February 28, 2003

STUDY COMPLETION DATE: July 8, 2003

SUBMITTED TO :

American Chemistry Council's
Brominated Flame Retardant Industry Panel
1300 Wilson Boulevard
Arlington, Virginia 22209

Wildlife International, Ltd.

8598 Commerce Drive
Easton, Maryland 21601
(410) 822-8600

- 2 -

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

SPONSOR: American Chemistry Council's Brominated Flame Retardant Industry Panel

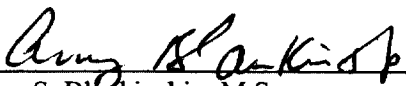
TITLE: Tetrabromobisphenol A: A 96-Hour Flow-Through Acute Toxicity Test with the Rainbow Trout (*Oncorhynchus mykiss*)

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 439A-123

STUDY COMPLETION: July 8, 2003

This study was conducted in compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency (40 CFR Parts 160 and/or 792, 17 August 1989); OECD Principles of Good Laboratory Practice (ENV/MC/CHEM (98)17); and Japan MAFF (11 NohSan, Notification No. 6283, Agricultural Production Bureau, 1 October 1999).

STUDY DIRECTOR:



Amy S. Blankinship, M.S.
Aquatic Biologist

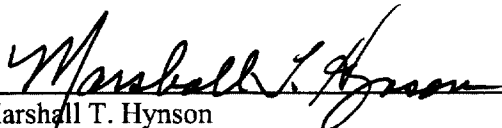
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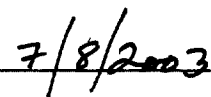
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QUALITY ASSURANCE STATEMENT

This study was examined for compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency (40 CFR Parts 160 and/or 792, 17 August 1989); OECD Principles of Good Laboratory Practice (ENV/MC/CHEM (98)17); and Japan MAFF (11 NohSan, Notification No. 6283, Agricultural Production Bureau, 1 October 1999). The dates of all inspections and audits and the dates that any findings were reported to the Study Director and Laboratory Management were as follows:

ACTIVITY:	DATE CONDUCTED:	DATE REPORTED TO:	
		STUDY DIRECTOR:	MANAGEMENT:
Test Substance Preparation	February 28, 2003	March 3, 2003	March 5, 2003
Matrix Fortification	March 3, 2003	March 3, 2003	March 5, 2003
Matrix Fortification	March 5, 2003	March 5, 2003	March 7, 2003
Observations & Water Chemistry	March 6, 2003	March 6, 2003	March 11, 2003
Protocol	March 10, 2003	March 10, 2003	March 12, 2003
Analytical Data & Draft Report	March 14 & 17, 2003	March 17, 2003	March 17, 2003
Biological Data & Draft Report	March 14, 2003	March 14, 2003	March 17, 2003
Final Report	July 2, 2003	July 2, 2003	July 2, 2003


Marshall T. Hynson
Quality Assurance Program Supervisor


Date

- 4 -

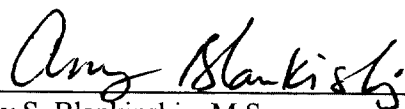
REPORT APPROVAL

SPONSOR: American Chemistry Council's Brominated Flame Retardant Industry Panel

TITLE: Tetrabromobisphenol A: A 96-Hour Flow-Through Acute Toxicity Test with the Rainbow Trout (*Oncorhynchus mykiss*)

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 439A-123

STUDY DIRECTOR :

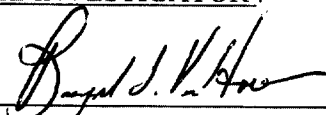


Amy S. Blankinship, M.S.
Aquatic Biologist

7-8-03

Date

PRINCIPAL INVESTIGATOR :

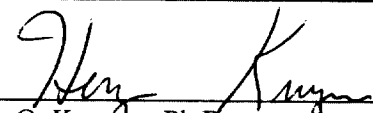


Raymond L. Van Hoven, Ph.D.
Scientist

7/8/03

Date


WILDLIFE INTERNATIONAL, LTD. MANAGEMENT :



Henry O. Krueger, Ph.D.
Director of Aquatic Toxicology/Terrestrial Plants and Insects

7/8/03

Date



Willard B. Nixon, Ph.D.
Director of Chemistry

7/8/03

Date

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SUMMARY

SPONSOR : American Chemistry Council's Brominated Flame Retardant Industry Panel

TITLE : Tetrabromobisphenol A: A 96-Hour Flow-Through Acute Toxicity Test with the Rainbow Trout (*Oncorhynchus mykiss*)

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER : 439A-123

TEST DATES :	Study Initiation :	February 28, 2003
	Experimental Start (OECD) :	February 28, 2003
	Experimental Start (EPA) :	March 3, 2003
	Biological Termination :	March 7, 2003
	Experimental Termination :	March 7, 2003

LENGTH OF EXPOSURE : 96 Hours

TEST ORGANISMS : Rainbow Trout (*Oncorhynchus mykiss*)

SOURCE OF TEST ORGANISMS : Thomas Fish Company
Anderson, California 96007

AGE OF TEST ORGANISMS : Juveniles

MEASUREMENTS OF 10 NEGATIVE CONTROL FISH :	Mean Wet Weight :	0.54 g	Range : 0.31 – 0.70 g
	Mean Total Length :	4.1 cm	Range : 3.5 – 4.7 cm

TEST CONCENTRATIONS :	<u>Nominal</u>	<u>Mean Measured</u>
	Negative Control	< LOQ
	Solvent Control	< LOQ
	1.2 mg a.i./L	1.1 mg a.i./L
	1.8 mg a.i./L	1.7 mg a.i./L

RESULTS : (Based on mean measured concentrations)

96-Hour LC50 :	1.1 mg a.i./L
95% Confidence Interval :	Not Applicable
No Mortality Concentration :	< 1.1 mg a.i./L
NOEC :	< 1.1 mg a.i./L

INTRODUCTION

This study was conducted by Wildlife International, Ltd. for the American Chemistry Council's Brominated Flame Retardant Industry Panel at the Wildlife International, Ltd. aquatic toxicology facility in Easton, Maryland. The in-life phase of the definitive toxicity test was conducted from March 3, to 7, 2003. Raw data generated by Wildlife International, Ltd. and a copy of the final report are filed under Project Number 439A-123 in archives located on the Wildlife International, Ltd. site.

OBJECTIVE

The objective of this study was to determine if the acute toxicity of tetrabromobisphenol A (TBBPA) to the rainbow trout, *Oncorhynchus mykiss*, during a 96-hour exposure period under flow-through test conditions would be >1.0 mg active ingredient (a.i.)/L.

EXPERIMENTAL DESIGN

Rainbow Trout were exposed to two test concentrations, a negative (dilution water) control, and a solvent (0.1 mL dimethyl formamide/L) control for 96 hours under flow-through conditions. Two replicate test chambers were maintained in each treatment and control group, with 10 fish in each test chamber for a total of 20 fish per concentration. Two nominal test concentrations were selected by the Sponsor to be assured that at least one mean measured concentration would be >1.0 mg a.i./L. Nominal test concentrations were 1.2 and 1.8 mg (a.i.)/L. Mean measured test concentrations were determined from samples of test water collected from each treatment and control group at the beginning of the test, at approximately 48 hours, and at test termination.

Delivery of the test substance was initiated approximately 66 hours prior to test initiation in order to achieve equilibrium of the test substance in the test chambers. The test organisms were impartially assigned to test chambers at test initiation. Observations of mortality and other signs of toxicity were made approximately 5.5, 24, 48, 72 and 96 hours after test initiation. The cumulative percent mortality observed in the treatment groups was used to estimate LC50 values at 24, 48, 72 and 96 hours. The no mortality concentration and the no-observed-effect-concentration (NOEC) were determined by visual interpretation of the mortality and observation data.

MATERIALS AND METHODS

The study was conducted based on the procedures outlined in the protocol, "Tetrabromobisphenol A: A 96-Hour Flow-Through Acute Toxicity Test with the Rainbow Trout (*Oncorhynchus mykiss*)" (Appendix 4). The protocol was based on procedures outlined in the OECD Guidelines for Testing of Chemicals, 203: *Fish, Acute Toxicity Test* (1); U.S. Environmental Protection Agency Series 850 – Ecological Effects Test Guidelines, OPPTS Number 850.1075, *Fish Acute Toxicity Test, Freshwater and Marine* (2); and ASTM Standard E729-88a: *Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates and Amphibians* (3).

Test Substance

The test substance used in the study consisted of a composite of TBBPA samples received from three manufacturers (Great Lakes Chemical Corporation (lot# 1008JE04B), Albermarle Corporation (lot# 25115T-1), and Bromine Compounds, Ltd. (lot# 010040)) between August 16 – 31, 2001. The composite sample was prepared by Wildlife International, Ltd. and assigned Wildlife International identification number 5754. Subsamples of the composite were shipped to Albermarle Corporation for characterization and purity analysis. The test substance, a white powder, was identified as: TBBPA composite; CAS Number 79-94-7. The reported purity was 99.17%, but the sponsor did not indicate an expiration date (Appendix 5). The test substance was stored under ambient conditions.

Test Organism

The rainbow trout, *Oncorhynchus mykiss*, was selected as the test species for this study. Rainbow trout are representative of an important group of aquatic vertebrates, and were selected for use in the test based upon past history of use in the laboratory. All fish used in the test were from the same source and year class. The fish were hatched on January 12, 2003 and were obtained in the swim-up stage from Thomas Fish Company, Anderson, California. Identification of the species was verified by the supplier.

The fish were held for at least 14 days prior to the test in water from the same source and at approximately the same temperature as used during the test. During the 14-day period preceding the test, water temperatures ranged from 10.5 to 12.0°C, measured with a hand-held liquid-in-glass thermometer. The pH of the water ranged from 8.2 to 8.5, measured with a Fisher Scientific Accumet

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Model 915 pH meter. Dissolved oxygen ranged from 9.4 to 10.3 mg/L ($\geq 87\%$ of saturation), measured with a Yellow Springs Instruments Model 51B dissolved oxygen meter. The fish were acclimated to test conditions for approximately 50 hours prior to test initiation. During the acclimation period, no mortalities occurred, and the fish showed no signs of disease or stress. At test initiation, the fish were collected from the acclimation tank and impartially distributed two at a time to the test chambers until each contained 10 fish.

During the holding period, the rainbow trout were fed daily a commercially-prepared diet supplied by Zeigler Brothers, Inc., Gardners, Pennsylvania. The fish were not fed for at least two days prior to the test or during the test.

The length of the longest fish measured at the end of the test was no more than twice the length of the shortest. The average total length of 10 negative control fish measured at the end of the test was 4.1 cm, with a range of 3.5 to 4.7 cm. The average wet weight (blotted dry) of 10 negative control fish measured at the end of the test was 0.54 grams, with a range of 0.31 to 0.70 grams. Loading was defined as the total wet weight of fish per liter of test water that passed through the test chamber in 24 hours, and was 0.060 g fish/L/day. Instantaneous loading was 0.36 g fish/L of test water present in the test chambers at any given time.

Dilution Water

The water used for culturing and testing was freshwater obtained from a well approximately 40 meters deep located on the Wildlife International, Ltd. site. The well water is characterized as moderately-hard water. The specific conductance, hardness, alkalinity and pH of the well water during the four-week period immediately preceding the test are presented in Appendix 1.

The well water was passed through a sand filter to remove particles greater than approximately 25 μm , and pumped into a 37,800-L storage tank where the water was aerated with spray nozzles. Prior to use, the water was filtered to 0.45 μm to remove microorganisms and particles. The results of periodic analyses performed to measure the concentrations of selected organic and inorganic constituents in the well water used by Wildlife International, Ltd. are presented in Appendix 2.

Test Apparatus

A continuous-flow diluter was used to deliver each concentration of the test substance, a solvent control and a negative control. A syringe pump (Harvard Apparatus, South Natick, Massachusetts) was used to deliver the two test substance stock solutions and dimethyl formamide (DMF) for the solvent control into mixing chambers assigned to each treatment and the solvent control. The syringe pump was calibrated prior to the test. The stock solutions were mixed with dilution water (Wildlife International, Ltd. well water) in the mixing chambers in order to obtain the desired test concentrations. The flow of dilution water to the mixing chambers was controlled by rotameters which were calibrated before the test. The flow of test water from each mixing chamber was split and allowed to flow into replicate test chambers. The proportion of test water that was split into each replicate was checked prior to the test to ensure that flow rates varied by no more than $\pm 10\%$ of the mean of the two replicates.

The diluter was adjusted so that each test chamber received approximately six volume additions of test water every 24 hours. The general operation of the diluter was checked visually at least two times per day during the test and at least once at the beginning and end of the test.

Test chambers were 25-L stainless steel aquaria filled with approximately 15 L of test water. The depth of the test water in a representative chamber was approximately 17.6 cm. The test chambers were covered with nylon mesh to prevent the rainbow trout from escaping. Test chambers were indiscriminately positioned in a temperature-controlled water bath set to maintain the desired test temperature. The water bath was enclosed in a plexiglass ventilation hood in order to minimize any potential for cross-contamination. The test chambers were labeled with the project number, test concentration and replicate.

Preparation of Test Concentrations

One stock solution was prepared for each of the two concentrations tested. A primary stock solution was prepared in DMF at a nominal concentration of 18 mg a.i./mL. An aliquot of the primary stock solution was proportionally diluted with DMF to prepare an additional stock solution at a concentration of 12 mg a.i./mL. The primary stock solution was mixed by inversion and sonication, and was clear and colorless in appearance. The additional stock was mixed by inversion and was clear and colorless in appearance. The two stock solutions were injected into the diluter mixing

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chambers (at a rate of 12.5 μ L/minute) where they were mixed with well water (at a rate of 125 mL/minute) to achieve the desired test concentrations. The solvent control was prepared by injecting DMF into the mixing chamber for the solvent control. The concentration of DMF in the solvent control and all TBBPA treatment groups was 0.1 mL/L. The test solutions appeared clear and colorless in the test chambers at test initiation and termination. However, a slight white precipitate on the mixing chambers was observed for the test solutions in the diluter mixing chambers at test initiation and also a surface slick in addition to the precipitation was observed at test termination. The test solutions were adjusted for active ingredient (99.17%).

Analytical Sampling

Samples were collected from both replicate test chambers in each treatment and control group at test initiation, at the approximate mid-point of the test, and at test termination to measure concentrations of the test substance. All samples were collected at mid-depth, placed in glass vials, and processed immediately for analysis.

Analytical Method

The analytical method used for the analysis of TBBPA in freshwater was developed at Wildlife International, Ltd. (Appendix 3). The analytical method consisted of dilution of the aqueous samples in 50% (v/v) methanol in NANOpure[®] water solution, and analysis by direct injection high performance liquid chromatography mass spectrometry (HPLC/MS).

Concentrations of TBBPA were determined by HPLC/MS using a Hewlett-Packard Model 1100 High Performance Liquid Chromatograph interfaced with a Perkin-Elmer SCIEX API 100 Mass Spectrometer. Chromatographic separations were achieved with a Keystone Betasil C₁₈ column (50 mm \times 2 mm, 3- μ m particle size) fitted with a Keystone Javelin C₁₈ guard column (20 mm \times 2 mm). A flow chart for the analysis of TBBPA is provided in Appendix 3.1, and typical instrumental parameters are summarized in Appendix 3.2.

Calibration standards of TBBPA, ranging in concentration from 0.0100 to 0.100 mg a.i./L, were prepared in 50% (v/v) methanol in NANOpure[®] water solution using a stock solution of TBBPA in methanol (Appendix 3.3). Linear regression equations were generated using the peak area for each standard versus the respective concentrations of the calibration standards. The concentration of TBBPA

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in the samples was determined by substituting the peak area responses into the applicable linear regression equation. An example of the calculations for a representative sample is included in Appendix 3.4.

The limit of quantitation (LOQ) for the freshwater analyses was set at 0.250 mg a.i./L, calculated as the product of the lowest calibration standard (0.0100 mg a.i./L) and the dilution factor of the matrix blank samples (25).

Quality control samples were prepared in freshwater and analyzed concurrently with test samples at each sampling interval to assess the performance of the analytical methodology. Three matrix blank samples were analyzed to determine possible interferences. No interferences were observed at or above the LOQ in the matrix blanks during the sample analyses (Appendix 3.5). Samples of freshwater were fortified at 1.00, 1.50, and 2.00 mg a.i./L using the appropriate fortification stock solution of TBBPA in methanol (Appendix 3.3), and were analyzed concurrently with the samples. The measured concentrations for the matrix fortification samples ranged from 85.1 to 104% of nominal concentrations (Appendix 3.5).

A representative calibration curve for TBBPA is presented in Appendix 3.6. Representative chromatograms of low and high-level calibration standards are presented in Appendices 3.7 and 3.8, respectively. A representative chromatogram of a matrix blank sample is presented in Appendix 3.9, and a representative chromatogram of a matrix fortification sample is presented in Appendix 3.10. A representative chromatogram of a test sample is presented in Appendix 3.11.

Environmental Conditions

Fluorescent light bulbs that emit wavelengths similar to natural sunlight (Colortone® 50) were used for illumination of the culture and test chambers during holding, acclimation and testing. A photoperiod of 16 hours of light and 8 hours of darkness was controlled with an automatic timer. A 30-minute transition period of low light intensity was provided when lights went on and off to avoid sudden changes in lighting. Light intensity at test initiation was 239 lux at the surface of the water of one representative test chamber.

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The target test temperature during the study was $12 \pm 1^{\circ}\text{C}$. Temperature was measured in each test chamber at the beginning and end of the test using a liquid-in-glass thermometer. Temperature also was measured continuously during the test in one negative control test chamber using a Fulscope ER/C Recorder, which was verified prior to test initiation with a liquid-in-glass thermometer. Dissolved oxygen and pH were measured in alternating test chambers at the beginning and end of the test and at approximately 24-hour intervals during the test. When a treatment replicate reached 100% mortality, measurements of DO, pH and temperature were made at that time and then discontinued. Hardness, alkalinity and specific conductance were measured in the dilution water at the beginning of the test.

Light intensity was measured using a SPER Scientific Model 840006C light meter. Dissolved oxygen was measured using a Thermo Orion Model 850Aplus dissolved oxygen meter, and measurements of pH were made using a Thermo Orion Model 525Aplus meter. Specific conductance was measured using a Yellow Springs Instrument Model 33 Salinity-Conductivity-Temperature meter. Hardness and alkalinity measurements were made by titration based on procedures in *Standard Methods for the Examination of Water and Wastewater* (4).

Observations

Observations were made periodically to determine the number of mortalities. The number of individuals exhibiting signs of toxicity or abnormal behavior also were evaluated. Observations were made approximately 5.5, 24, 48, 72 and 96 hours after test initiation.

Statistical Analyses

The mortality data at 48, 72 and 96 hours were analyzed using the computer program of C. E. Stephan (5). The program was designed to calculate the LC50 value and the 95% confidence interval by probit analysis, the moving average method, and binomial probability with nonlinear interpolation (6, 7, 8). Due to insufficient mortality at 24-hours in this study, a LC50 value at 24-hours could not be calculated. The binomial probability method was used to calculate the 48, 72, and 96-hour LC50 values. Also, due to the mortality in the treatment groups, 95% confidence intervals could not be calculated for the 72 and 96-hour LC50 values. The no mortality concentration and the no-observed-effect-concentration (NOEC) were determined by visual interpretation of the mortality and observation data.

RESULTS AND DISCUSSION

Measurement of Test Concentrations

Nominal concentrations selected for use in this study were 1.2 and 1.8 mg a.i./L. Results of analyses to measure concentrations of TBBPA in the samples collected during the test ranged from 75 to 106% of nominal (Table 1). When the measured concentrations of the test samples collected at test initiation, at approximately 48 hours and at test termination were averaged, the mean measured concentrations for the study were 1.1 and 1.7 mg a.i./L, representing 92 and 94% of nominal concentrations, respectively. The results of the study were based on the mean measured test concentrations.

Observations and Measurements

Measurements of temperature, dissolved oxygen and pH of the water in each test chamber are presented in Table 2. Water temperatures were within the $12 \pm 1^{\circ}\text{C}$ range established for the test. Dissolved oxygen (DO) concentrations in the treatment groups remained at or above 8.8 mg/L (81% of saturation) throughout the test. Measurements of pH ranged from 8.0 to 8.2. The measurements of hardness, alkalinity and specific conductance in the dilution water at test initiation were typical of Wildlife International, Ltd. well water (Table 3).

Daily observations for mortality and signs of toxicity during the test are presented in Table 4. Rainbow trout in the negative and solvent control groups appeared normal throughout the test. Percent mortality at test termination in the 1.1 and 1.7 mg a.i./L treatment groups was 45 and 100%, respectively. Rainbow trout remaining in the 1.1 mg a.i./L treatment group at test termination were observed to be either lying on the bottom of the test chamber or showing signs of lethargy compared to the controls. Consequently, the no-mortality concentration and the NOEC were < 1.1 mg a.i./L. LC50 values at 24, 48, 72 and 96 hours were calculated from the mortality data and are presented in Table 5.

CONCLUSIONS

Rainbow trout, *Oncorhynchus mykiss*, were exposed to two concentrations of Tetrabromobisphenol A (TBBPA) under flow-through conditions for 96 hours. The 96-hour LC50 value was 1.1 mg a.i./L, but 95% confidence intervals could not be calculated. The 96-hour no-mortality concentration and the NOEC were both < 1.1 mg a.i./L, the lowest test concentration tested.

REFERENCES

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- 2 **U.S. Environmental Protection Agency.** 1996. *Fish Acute Toxicity Test, Freshwater and Marine.* Series 850 – Ecological Effects Test Guidelines (*draft*), OPPTS Number 850.1075:
- 3 **ASTM Standard E729-88a.** 1994. *Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians.* American Society for Testing and Materials.
- 4 **APHA, AWWA, WPCF.** 1998. *Standard Methods for the Examination of Water and Wastewater.* 20th Edition, American Public Health Association. American Water Works Association. Water Pollution Control Federation, New York.
- 5 **Stephan, C.E.** 1978. U.S. EPA, Environmental Research Laboratory, Duluth, Minnesota. Personal communication.
- 6 **Thompson, W.R.** 1947. *Bacteriological Reviews.* Vol. II, No. 2. Pp 115-145.
- 7 **Stephan, C.E.** 1977. "Methods for Calculating an LC50", *Aquatic Toxicology and Hazard Evaluations.* American Society for Testing and Materials. Publication Number STP 634, pp 65-84.
- 8 **Finney, D.J.** 1971. *Statistical Methods in Biological Assay.* Second edition. Griffin Press, London.

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Table 1

Measured Concentrations of Tetrabromobisphenol A (TBBPA) in Test Samples

Nominal Test Concentration (mg a.i./L)	Sample ID (439A-123-)	Sampling Time (Hours)	Measured Concentration (mg a.i./L) ^{1,2}	Percent of Nominal ²	Mean Measured Concentration (mg a.i./L)	Mean Percent of Nominal
Negative Control 0.0	1	0	< LOQ	--	--	--
	2	0	< LOQ	--	--	--
	9	48	< LOQ	--	--	--
	10	48	< LOQ	--	--	--
	17	96	< LOQ	--	--	--
	18	96	< LOQ	--	--	--
Solvent Control 0.0	3	0	< LOQ	--	--	--
	4	0	< LOQ	--	--	--
	11	48	< LOQ	--	--	--
	12	48	< LOQ	--	--	--
	19	96	< LOQ	--	--	--
	20	96	< LOQ	--	--	--
1.2	5	0	0.978	81.5	1.1	92
	6	0	0.902	75.2		
	13	48	1.15	95.6		
	14	48	1.14	94.8		
	21	96	1.15	95.5		
	22	96	1.13	94.2		
1.8	7	0	1.60	89.1	1.7	94
	8	0	1.35	75.2		
	15	48	1.91	106		
	16	48	1.90	106		
	--	96 ³	--	--		
	--	96 ³	--	--		

¹ The limit of quantitation (LOQ) was 0.250 mg a.i./L calculated as the product of the lowest calibration standard (0.0100 mg a.i./L) and the dilution factor of the matrix blanks (25).

² Results were generated using MacQuan version 1.6 software. Manual calculations may differ slightly.

³ Samples not collected due to 100% mortality at 48 hours.

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Table 2

Temperature, Dissolved Oxygen and pH of Water in the Test Chambers

Mean Measured Test Concentration (mg a.i./L)	Replicate	0 Hour		24 Hours		48 Hours		72 Hours		96 Hours	
		Temp ¹ (°C)	DO ² (mg/L)	pH	DO (mg/L)	pH	DO (mg/L)	pH	DO (mg/L)	Temp (°C)	DO (mg/L)
Negative Control	A	12.3	9.6	8.2	--	--	9.1	8.0	--	11.9	9.2
	B	12.3	--	--	9.3	8.0	--	--	8.8	11.8	--
Solvent Control	A	12.3	9.4	8.2	--	--	9.2	8.1	--	11.9	9.1
	B	12.3	--	--	9.3	8.1	--	--	8.9	11.9	--
1.1	A	12.0	9.4	8.2	--	--	9.1	8.1	--	11.6	9.3
	B	12.0	--	--	9.2	8.1	--	--	9.0	11.5	--
1.7	A	12.2	9.3	8.2	--	--	9.1 ³	8.1 ³	--	-- ⁴	-- ⁴
	B	12.2	--	--	9.1	8.1	9.1 ³	8.1 ³	-- ⁴	-- ⁴	--

¹ Manual temperature measurements. Temperature measured continuously during the test ranged from approximately 11.5 to 12.5°C, measured to the nearest 0.5°C.² A dissolved oxygen concentration of 8.1 mg/L represents 75% saturation at 12°C in freshwater.³ Temperature measured in Replicate A and B was 11.9°C and 11.9°C, respectively. Temperature, DO, and pH measured in each replicate due to 100% mortality.⁴ Measurement discontinued due to 100% mortality.

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Table 3

Hardness, Alkalinity and Specific Conductance
Measured in Dilution Water at Test Initiation

Parameter	Day 0
Hardness (mg/L as CaCO ₃)	128
Alkalinity (mg/L as CaCO ₃)	181
Specific Conductance (µmhos/cm)	270

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Table 4

Cumulative Percent Mortality and Observed Effects

Mean Measured Test Concentration (mg a.i./L.)	Rep.	No. Exposed	5.5 Hours		24 Hours		48 Hours		72 Hours		96 Hours		Cumulative Percent Mortality
			No. Dead ¹	Effects ²	No. Dead	Effects	No. Dead	Effects	No. Dead	Effects	No. Dead	Effects	
Negative Control	A	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0
	B	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	
Solvent Control	A	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0
	B	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	
1.1	A	10	0	10 AN	0	9 C, 1 R	0	1 AN, 3 C, 3 R, 3 N	3	2 C, 4 R, 1 N	5	2 C, 3 R	45
	B	10	0	10 AN	0	2 AN, 8 C	0	8 C, 1 R, 1 N	3	2 C, 3 R, 2 N	4	3 C, 3 R	
1.7	A	10	0	5 AN, 5 C	1	9 R	10	--	10	--	10	--	100
	B	10	0	3 AN, 7 C	1	9 R	10	--	10	--	10	--	

¹ Cumulative number of dead fish.

² Observed Effects: AN = appear normal; C = lethargic; R = lying on bottom of test chamber, N = loss of equilibrium.

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Table 5

LC50 Values

Time	LC50 (mg a.i./L)	95% Confidence Interval (mg a.i./L)	Statistical Method
24 Hours	> 1.7	-- ¹	NA ²
48 Hours	1.4	1.1 – 1.7	Binomial Probability
72 Hours	1.2	-- ¹	Binomial Probability
96 Hours	1.1	-- ¹	Binomial Probability
¹ 95% confidence limits could not be calculated from the data.			
² NA = not applicable; <50% mortality/immobility precluded statistical calculation of an EC50 value.			

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Appendix 1

Specific Conductance, Hardness, Alkalinity and pH of Well Water Measured
During the 4-Week Period Immediately Preceding the Test

Parameter	Mean	Range
Specific Conductance (μ mhos/cm)	310 (N = 4)	310 – 310
Hardness (mg/L as CaCO ₃)	131 (N = 4)	124 – 140
Alkalinity (mg/L as CaCO ₃)	180 (N = 4)	170 – 184
pH	8.2 (N = 4)	8.1 – 8.4

Appendix 2

Analyses of Pesticides, Organics and Metals in Wildlife International, Ltd. Well Water¹

Pesticides and Organics			
Component	Measured Concentration (ppb or ng/g)	Component	Measured Concentration (ppb or ng/g)
Aldicarb sulfone	< 50	Isofenphos	< 50
Aldicarb sulfoxide	< 50	Leptophos	< 50
Azinphos-ethyl	< 50	Linuron	< 50
Azinphos-methyl	< 50	Methidathion	< 50
Bifenox	< 50	Methiocarb	< 50
Bitertanol	< 50	Methomyl	< 50
Bromacil	< 50	Methoxychlor	< 250
Bromoxynil octanoic acid ester	< 50	Mirex	< 50
Captafol	< 50	Monocrotophos	< 50
Carbaryl	< 50	Myclobutanil	< 50
3-Hydroxy Carbofuran	< 50	Napropamide	< 50
Carbofuran	< 50	Norflurazon	< 50
Carbophenothion	< 50	Oxadiazon	< 50
cis-Chlordane	< 50	Oxamyl	< 50
trans-Chlordane	< 50	Oxyfluorfen	< 50
Chlorfenson	< 50	Paraoxon	< 50
trans-Chlorfenvinphos	< 50	cis-Permethrin	< 50
Chlorobenzilate	< 50	Perthane	< 50
Chloropropylate	< 50	Phosalone	< 50
Chloroxuron	< 50	Phosphamidon	< 50
Coumaphos	< 50	Piperalin	< 50
Crotoxyphos	< 50	Profenfos	< 50
Cyanazine	< 50	Promecarb	< 50
Cyfluthrin I	< 50	Propanil	< 50
Cypermethrin I	< 50	Propargite	< 50
o,p'-DDD	< 50	Propoxur	< 50
p,p'-DDE	< 50	Pyrethrin I	< 50
p,p'-DDD	< 50	Quinalphos	< 50
o,p'-DDT	< 50	Quinomethionate	< 50
p,p'-DDT	< 250	Quizalofop-ethyl	< 50
DEF	< 50	Sulprofos	< 50
Diclofop methyl	< 50	Tetrachlorovinphos	< 50
Dicrotophos	< 50	Tetradifon	< 50
Dieldrin	< 50	Thiobendazole	< 50
Diphenamid	< 50	Tilt I	< 50
Diuron	< 50	Tilt II	< 50
Endosulfan II	< 50	Trimethyl carbamate	< 50
Endrin	< 50		
Endrin ketone	< 50		
EPN	< 50		
Ethion	< 50		
Fenamiphos	< 50		
Fenarimol	< 50		
Fenobucarb	< 50		
Fenpropathrin	< 50		
Fensulfothion	< 50		
Fluzifop-P-butyl	< 50		

¹Analyses performed by Exygen Research on samples collected on July 31, 2002.

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Appendix 2 (Continued)Analyses of Pesticides, Organics and Metals in Wildlife International, Ltd. Well Water¹

Metals			
	(ppm or mg/L)		(ppm or mg/L)
Aluminum	< 0.204	Manganese	< 0.0153
Arsenic	< 0.0102	Mercury	< 0.0002
Beryllium	< 0.0051	Molybdenum	< 0.0005
Cadmium	< 0.0051	Nickel	< 5.1
Calcium	28.2	Potassium	5.45
Chromium	< 0.0102	Selenium	0.009
Cobalt	< 5.1	Silver	< 0.0102
Copper	< 0.0255	Sodium	18.6
Iron	< 5.1	Zinc	< 0.0204
Magnesium	11.6		

¹Analyses performed by Exygen Research on samples collected on July 31, 2002.

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Appendix 3

The Analysis of Tetrabromobisphenol A (TBBPA) in Freshwater

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Appendix 3.1

Analytical Method Flowchart for the Analysis of TBBPA in Freshwater

**METHOD OUTLINE FOR THE ANALYSIS OF
TBBPA IN FRESHWATER**

Prepare quality control samples in freshwater using gas-tight syringes and volumetric flasks.
The matrix blank will be freshwater.



Dilute submitted samples and quality control samples with 50% (v/v) methanol NANOpure® water
solution using gas-tight syringes and volumetric flasks.



Transfer samples to autosampler vials and submit samples for
HPLC/MS analysis.

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Appendix 3.2

Typical HPLC/MS Operational Parameters

INSTRUMENT:	Hewlett-Packard Model 1100 High Performance Liquid Chromatograph with a Perkin-Elmer SCIEX API 100 Mass Spectrometer configured with a Heated Nebulizer ion source. Operated in negative selective ion monitoring mode.
ANALYTICAL COLUMN:	Keystone Betasil C ₁₈ column (50 mm × 2 mm, 3-μm particle size)
GUARD COLUMN:	Keystone Javelin C ₁₈ column (20 mm × 2 mm)
OVEN TEMPERATURE:	40°C
STOP TIME:	5.00 minutes
FLOW RATE:	0.250 mL/minute
MOBILE PHASE:	Solvent A: 0.1% formic acid in NANOpure [®] water (20%) Solvent B: Methanol (80%)
INJECTION VOLUME:	100 μL
TBBPA RETENTION TIME:	Approximately 3.4 minutes
TBBPA MONITORED MASS:	542.7 amu

Appendix 3.3**Analytical Stocks and Standards Preparation**

A primary stock solution of TBBPA was prepared by weighing 0.1008 g of the test substance on an analytical balance. The test substance (99.17% purity) was transferred to a 100-mL volumetric flask and brought to volume using methanol. The primary stock solution contained 1.00 mg a.i./mL of TBBPA. Secondary stocks (0.100, 0.0100, and 0.00100 mg a.i./mL TBBPA in methanol) were prepared from the primary stock by serial dilution. The primary and secondary stocks were used to fortify the quality control samples and to prepare calibration standards. Calibration standards for TBBPA were prepared in 50% (v/v) methanol in NANOpure® water solution. The following shows the dilution scheme for the calibration standards.

Stock Concentration (mg a.i./mL)	Aliquot (mL)	Final Volume (L)	Standard Concentration (mg a.i./L)
0.0100	0.100	0.100	0.0100
0.0100	0.250	0.100	0.0250
0.0100	0.500	0.100	0.0500
0.0100	0.750	0.100	0.0750
0.0100	1.00	0.100	0.100

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Appendix 3.4**Example Calculations for a Representative Sample**

The analytical result and percent recovery for sample number 439A-123-5, nominal concentration of 1.2 mg a.i./L, were calculated using the following equations:

$$\text{TBBPA (mg a.i./L) in sample} = \frac{\text{Peak area} - (\text{Y-intercept})}{\text{Slope}} \times \text{Dilution factor}$$

Peak Area = 589965

Y-intercept = 2567.6514

Slope = 15013852

Initial Volume (V_i): = 0.200 mL

Final Volume (V_f): = 5.00 mL

Dilution Factor (V_f/V_i): = 25

$$\text{TBBPA (mg a.i./L) in sample} = \frac{589965 - (2567.6514)}{15013852} \times 25$$

$$= 0.978 \text{ mg a.i./L}$$

$$\text{Percent of nominal concentration} = \frac{\text{TBBPA in sample (mg a.i./L)}}{\text{TBBPA nominal concentration (mg a.i./L)}} \times 100$$

$$= \frac{0.978}{1.2} \times 100$$

$$= 81.5\%$$

Results were generated using MacQuan version 1.6 software. Manual calculations may differ slightly.

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Appendix 3.5

Quality Control Samples of TBBPA in Freshwater

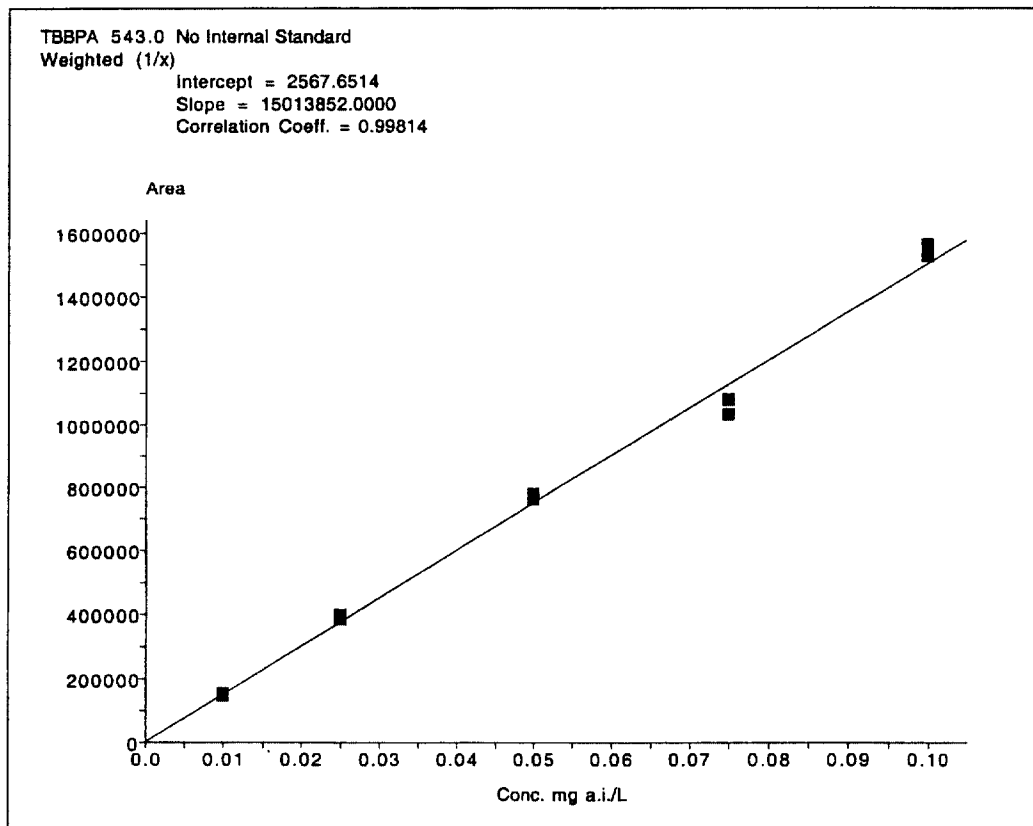
Sample Number	Concentration (mg a.i./L)		Percent Recovery ²
	Fortified	Measured ^{1,2}	
439A-123-MAB-1	0.0	< LOQ	--
439A-123,124-MAB-2	0.0	< LOQ	--
439A-123,124-MAB-3	0.0	< LOQ	--
439A-123-MAS-1	1.00	0.884	88.4
439A-123-MAS-2	1.50	1.28	85.1
439A-123-MAS-3	2.00	2.03	101
439A-123,124-MAS-4	1.00	0.994	99.4
439A-123,124-MAS-5	1.50	1.50	100
439A-123,124-MAS-6	2.00	2.08	104
439A-123,124-MAS-7	1.00	1.00	100
439A-123,124-MAS-8	1.50	1.51	100
439A-123,124-MAS-9	2.00	2.02	101

¹ The limit of quantitation (LOQ) was 0.250 mg a.i./L calculated as the product of the lowest calibration standard (0.0100 mg a.i./L) and the dilution factor of the matrix blanks (25).

² Results were generated using MacQuan version 1.6 software. Manual calculations may differ slightly.

Appendix 3.6

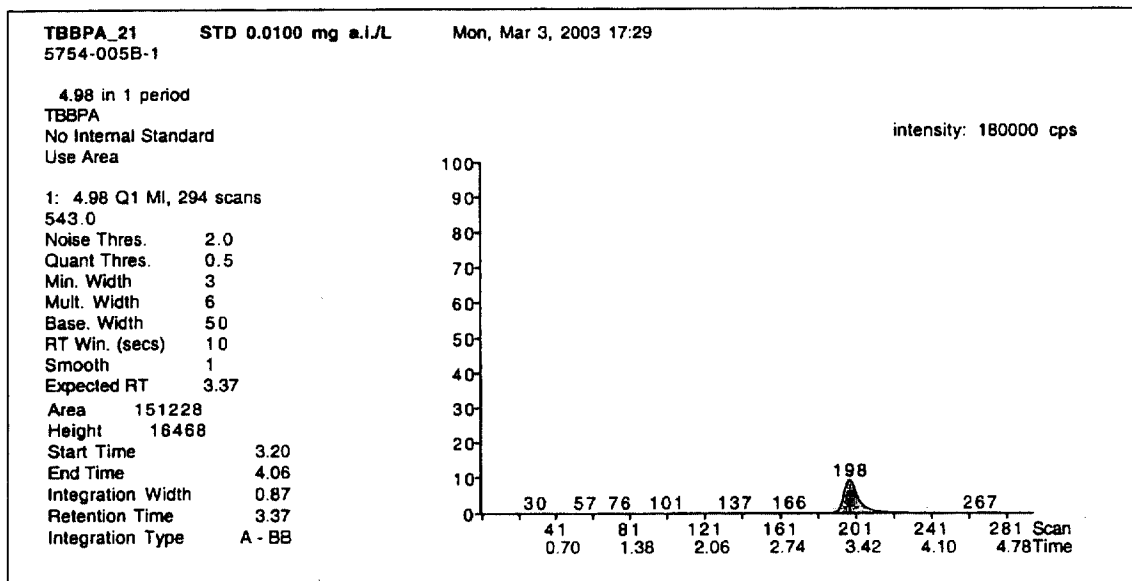
Representative Calibration Curve for TBBPA



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Appendix 3.7

Representative Chromatogram of a Low-level TBBPA Calibration Standard

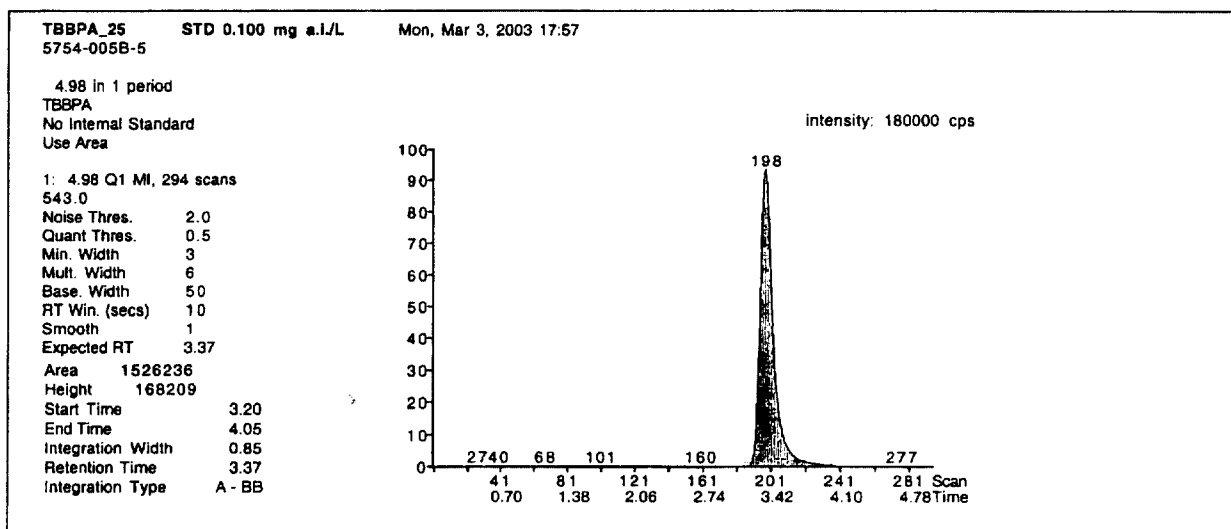


Nominal concentration: 0.0100 mg a.i./L

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Appendix 3.8

Representative Chromatogram of a High-level TBBPA Calibration Standard

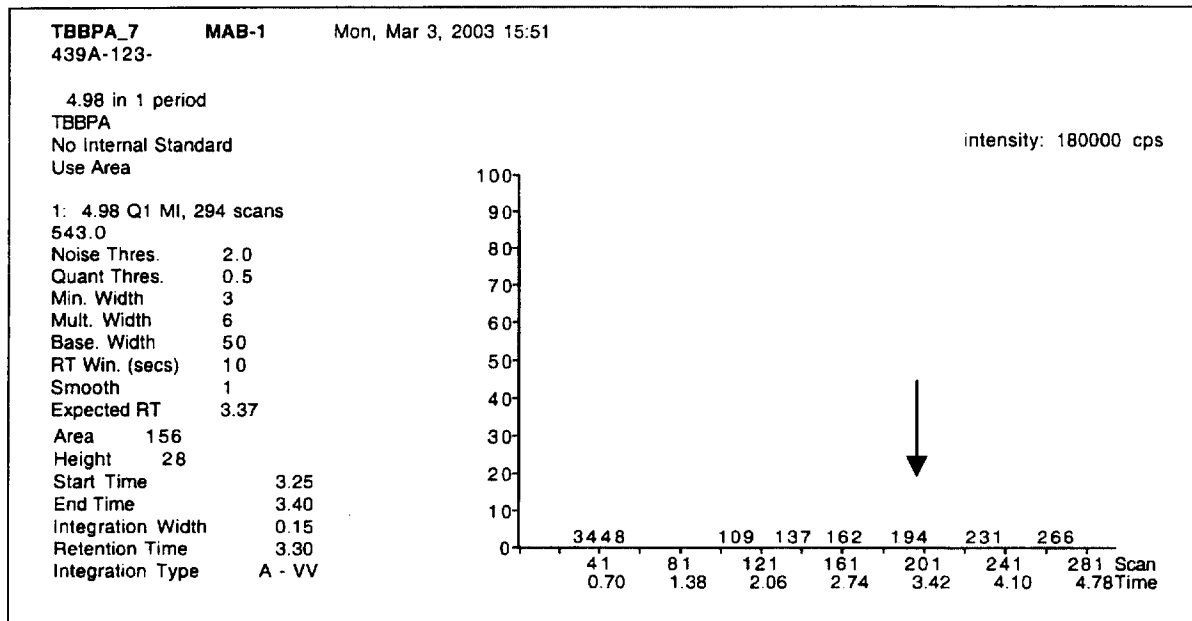


Nominal concentration: 0.100 mg a.i./L

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Appendix 3.9

Representative Chromatogram of a Matrix Blank Sample



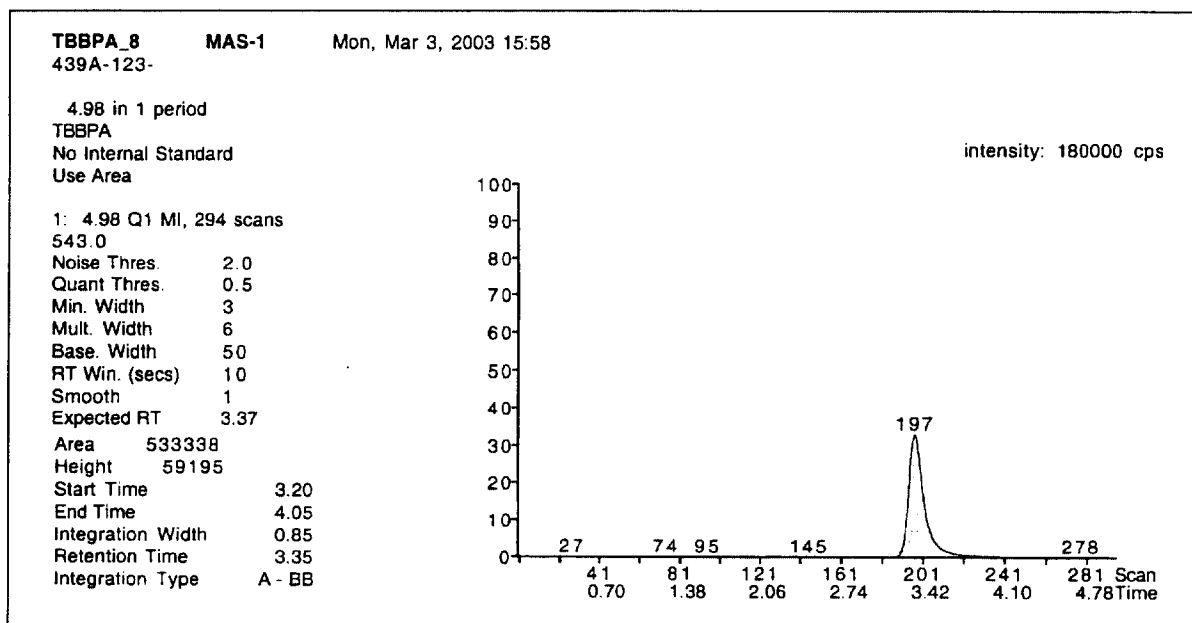
Sample number 439A-123-MAB-1. Dilution factor = 25X.

The arrow indicates the approximate retention time of TBBPA.

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Appendix 3.10

Representative Chromatogram of a Matrix Fortification Sample



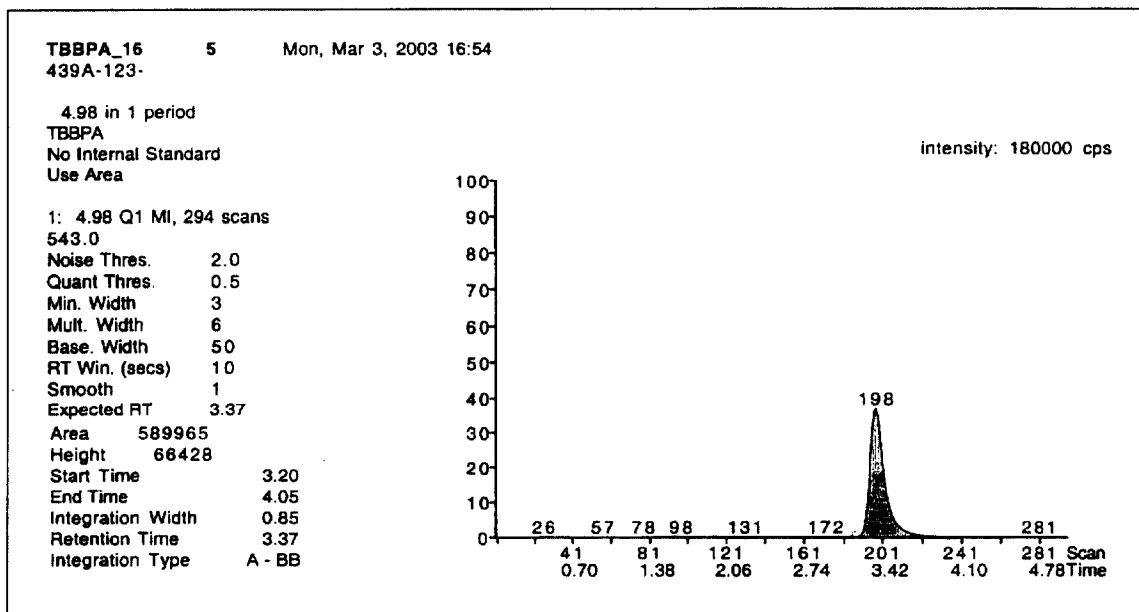
Sample number: 439A-123-MAS-1, nominal concentration 1.00 mg a.i./L.

Dilution factor = 25X.

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Appendix 3.11

Representative Chromatogram of a Test Sample



Sample number: 439A-123-5, Day 0, nominal concentration 1.2 mg a.i./L.

Dilution factor = 25X.

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Appendix 4

Protocol and Amendment

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PROTOCOL

TETRABROMOBISPHENOL A: A 96-HOUR FLOW-THROUGH ACUTE TOXICITY
TEST WITH THE RAINBOW TROUT (*Oncorhynchus mykiss*)

OECD Guideline 203

U.S. EPA OPPTS Number 850.1075

Submitted to

American Chemistry Council's
Brominated Flame Retardant Industry Panel
1300 Wilson Boulevard
Arlington, Virginia 22209

Wildlife International, Ltd.

8598 Commerce Drive
Easton, Maryland 21601
(410) 822-8600

February 13, 2003

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**TETRABROMOBISPHENOL A: A 96-HOUR FLOW-THROUGH ACUTE TOXICITY
TEST WITH THE RAINBOW TROUT (*Oncorhynchus mykiss*)**

SPONSOR: American Chemistry Council's
Brominated Flame Retardant Industry Panel
1300 Wilson Boulevard
Arlington, Virginia 22209

SPONSOR'S REPRESENTATIVE: Ms. Wendy Sherman

TESTING FACILITY: Wildlife International, Ltd.
8598 Commerce Drive
Easton, Maryland 21601

STUDY DIRECTOR: Amy Blankinship, Biologist
Wildlife International, Ltd.

LABORATORY MANAGEMENT: Henry O. Krueger, Ph.D.
Director of Aquatic Toxicology/Terrestrial Plants & Insects

FOR LABORATORY USE ONLY

Proposed Dates:	
Experimental Start Date: <u>3/3/03</u>	Experimental Termination Date: <u>3/7/03</u>
Project No.: <u>439A-123</u>	
Test Concentrations: <u>Negative Control, Solvent Control (0.1 mL DMF/L), 1.2 mg/L, 1.8 mg/L</u>	
Test Substance No.: <u>5754</u> Reference Substance No. (if applicable): <u>-</u>	

PROTOCOL APPROVAL

<u>Amy Blankinship</u> STUDY DIRECTOR	<u>2/28/03</u> DATE
<u>H. Krueger</u> LABORATORY MANAGEMENT	<u>2/28/03</u> DATE
<u>Susan Lewis for Wendy Sherman</u> SPONSOR'S REPRESENTATIVE	<u>2/26/03</u> DATE

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Wildlife International, Ltd.

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INTRODUCTION

Wildlife International, Ltd. will conduct a flow-through acute toxicity test with the rainbow trout (*Oncorhynchus mykiss*) for the Sponsor at the Wildlife International, Ltd. aquatic toxicology facility in Easton, Maryland. The study will be performed based on procedures in *OECD Guideline for Testing of Chemicals 203: Fish Acute Toxicity Test* (1); U.S. EPA Series 850 - Ecological Effects Test Guidelines OPPTS Number 850.1075: *Fish Acute Toxicity Test, Freshwater and Marine* (2); and ASTM Standard E729-88a: *Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates and Amphibians* (3). Raw data for all work performed at Wildlife International, Ltd. and a copy of the final report will be filed by project number in archives located on the Wildlife International, Ltd. site or at an alternative location to be specified in the final report.

OBJECTIVE

The objective of this study is to determine the acute effects of Tetrabromobisphenol A (TBBPA) on the rainbow trout (*Oncorhynchus mykiss*) during a 96-hour exposure period under flow-through test conditions.

EXPERIMENTAL DESIGN

Rainbow trout will be exposed to two test concentrations, (1.2 and 1.8 ppm), a negative (dilution water) control and a solvent control for 96 hours. Two replicate test chambers will be maintained in each treatment and control group, with 10 rainbow trout in each chamber for a total of 20 rainbow trout per test concentration.

Nominal test concentrations were selected in consultation with the Sponsor based upon information such as known toxicity data, physical/chemical properties of the test substance or other relevant information. Water samples will be collected from appropriate test chambers at specified intervals for analysis of the test substance. Results of analyses will be used to calculate mean measured test concentrations.

To control bias, rainbow trout will be impartially assigned to exposure chambers at test initiation. No other potential sources of bias are expected to affect the results of the study. Observations of mortality and other clinical signs will be made throughout the 96-hour test period. Cumulative percent

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mortality observed in the treatment groups will be used to calculate, when possible, LC50 values at 24, 48, 72 and 96 hour intervals. The no mortality concentration and the no-observed-effect concentration (NOEC) will be determined by visually interpreting the clinical observation data.

MATERIALS AND METHODS**Test Substance**

The test substance consisted of a composite of TBBPA samples received from three manufacturers. The material's identity and date received from each of the manufacturers is given below:

<u>Manufacturer</u>	<u>Lot/Batch</u>	<u>Date Received</u>	<u>Wildlife International Ltd. Identification Number</u>
Great Lakes Chemical Corporation	1008JE04B	August 16, 2001	5722
Albemarle Corporation	25115T-1	August 16, 2001	5721
Bromine Compounds, Ltd.	010040	August 31, 2001	5733

The composite test substance was assigned Wildlife International Ltd. identification number 5754 and was stored under ambient conditions. Subsamples of the composite test substance were shipped to Albemarle Corporation for characterization and purity analyses. The results of the analyses indicated the composite test substance was homogeneous and contained TBBPA with a purity of 99.17%. The test substance was stored at room temperature.

The Sponsor is responsible for all information related to the test substance and agrees to accept any unused test substance and/or test substance containers remaining at the end of the study.

Preparation of Test Concentrations

The test substance will be administered to the test organism in water. This route of administration was selected because it represents the most likely route of exposure to aquatic organisms.

The test substance will be mixed directly with dilution water or may be first mixed with a solvent. If a solvent is used, the test substance will be dissolved in the solvent to form a stock solution that will subsequently be added to dilution water. Reverse osmosis water will be the solvent of choice, although dimethyl formamide, triethylene glycol, methanol, ethanol or acetone may be used. If an organic solvent is required, a solvent control will be included in the test in addition to a negative (dilution water) control. The concentration of the organic solvent will not exceed 0.1 mL/L, when possible. The organic solvent

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concentration in the solvent control will be equal to the highest solvent concentration in test chambers containing the test substance.

Test Organism

The rainbow trout (*Oncorhynchus mykiss*) has been selected as the test species for this study. Rainbow trout are representative of an important group of aquatic vertebrates, and have been selected for use in the test based upon past use history in the laboratory. Fish will be from the same source and year class, and the length of the longest fish measured will be no more than twice that of the shortest. Fish will weigh between 0.1 and 3.0 grams (wet weight blotted dry), and the total weight in each test chamber will not exceed 0.5 grams per liter of solution passing through the chamber in 24 hours nor 5 g/L at any time. The recommended total length of fish is 5.0 ± 1.0 cm. Total lengths and wet weights of the individual fish in one negative control replicate will be measured at the end of the test and will be considered representative of the length and weight of all fish used in the study. Fish and/or fish eggs will be obtained from a supplier or hatchery, and the identity of the species will be verified by the supplier, or by Wildlife International, Ltd. personnel using appropriate taxonomic keys, such as Eddy (4).

Rainbow trout will be held for at least 14 days prior to the test in water from the same source and at approximately the same temperature as used during the test. Variations in water temperature will not exceed 3°C in any 72-hour period during holding, and dissolved oxygen must be > 80% of saturation during holding. If mortality of the test fish exceeds 3%, or the fish show signs of disease or stress during a two-day period immediately preceding the test, they will be euthanized or held for an additional 14-day period to ensure that the fish are healthy. At test initiation, the rainbow trout will be collected from holding or acclimation tanks and transferred to the test chambers.

During the holding period, the test fish will be fed at least once daily. The diet will consist of live or frozen brine shrimp nauplii (*Artemia sp.*), frozen brine shrimp, and/or commercial food. Fish will not be fed for at least two days prior to the test or during the test. Specifications for acceptable levels of contaminants in fish diets have not been established. However, there are no known levels of contaminants reasonably expected to be present in the diet that are considered to interfere with the purpose or conduct of the test.

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Dilution Water

Water used for the holding and testing of rainbow trout will be obtained from a well approximately 40 meters deep located on the Wildlife International, Ltd. site. The water will be passed through a sand filter and pumped into a 37,800-L storage tank where the water will be aerated with spray nozzles. Prior to use the water will be filtered to 0.45 μm in order to remove fine particles. Water used for holding and testing is characterized as moderately hard. Typical values for hardness, alkalinity, pH and specific conductance are approximately:

Hardness, mg/L as CaCO_3	145
Alkalinity, mg/L as CaCO_3	190
pH	8.1
Specific Conductance, $\mu\text{mhos/cm}$	330

Hardness, alkalinity, pH and specific conductance will be measured weekly to monitor the consistency of the well water. Means and ranges of the measured parameters for the four-week period preceding the test will be provided in the final report. Analyses will be performed at least once annually to determine the concentrations of selected organic and inorganic constituents of the well water and results of the most recent GLP-compliant analyses will be summarized in the final report.

Test Apparatus

A continuous-flow diluter will be used to provide each concentration of the test substance, a negative (dilution water) control, and a solvent control, when necessary. A syringe pump, peristaltic pump, or a similar device will be used to deliver the test substance to mixing chambers where the test substance will be mixed with dilution water. The flow of dilution water into each mixing chamber will be controlled using rotameters. After mixing, test solutions will be split to each replicate chamber. The proportion of water split to each replicate will be checked prior to the test to ensure that these flow rates vary by no more than $\pm 10\%$ of the mean of the two replicates. Test chambers will be 25-L, Teflon®-lined polyethylene or stainless steel chambers filled with approximately 15 L of water. Test chambers will be indiscriminately positioned in a temperature-controlled water bath designed to maintain a temperature of $12 \pm 1^\circ\text{C}$. The water bath will be enclosed in a plexiglass ventilation hood in order to minimize potential cross-contamination between test systems. Test chambers will be labelled with the project number, test concentration and replicate.

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In tests where solvent controls are required, the solvent will be injected into a mixing chamber where it will be diluted to the appropriate concentration with dilution water. The concentration of solvent in the solvent control will be equal to that in the highest treatment level.

The diluter will be adjusted so that each test chamber receives at least 5 volume additions of test solution every 24 hours. Test substance stock delivery pumps and rotameters will be calibrated before each study, and the delivery of test substance to test chambers will begin at least 4 hours prior to the test in order to establish equilibrium concentrations of the test substance. The general operation of the diluter will be checked visually at least two times per day during the test and at least once at the beginning and end of the test.

Environmental Conditions

Lighting used to illuminate the cultures and test chambers during holding, acclimation, and testing will be provided by fluorescent tubes that emit wavelengths similar to natural sunlight (e.g., Colortone® 50). A photoperiod of 16 hours of light and 8 hours of dark will be controlled with an automatic timer. A 30-minute transition period of low light intensity will be provided when lights go on and off to avoid sudden changes in light intensity. Light intensity will be measured at test initiation with a SPER Scientific Ltd. light meter or equivalent.

The target test temperature will be $12 \pm 1^\circ\text{C}$. Temperature will be measured in all replicates at the beginning and end of the test using a liquid-in-glass thermometer. Temperature also will be measured with a continuous recorder in one negative control chamber. Recorder measurements will be verified with a liquid-in-glass thermometer prior to test initiation.

Dissolved oxygen will be measured in alternate replicates of each treatment and control group at test initiation and at approximately 24-hour intervals thereafter using a Thermo Orion Model 850Aplus dissolved oxygen meter, or equivalent. In the event that dissolved oxygen levels fall below 75% saturation, dissolved oxygen measurements will be made in every test chamber and appropriate actions will be taken after consultation with the Sponsor. Measurements of pH will be made in alternate replicates of each treatment and control group at test initiation and at approximately 24-hour intervals thereafter using a Thermo Orion Model 720Aplus pH meter, or equivalent. If a treatment replicate reaches 100% mortality, dissolved oxygen, pH, and temperature measurements will be taken in that replicate at that time, and then discontinued.

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Hardness, alkalinity, and specific conductance will be measured in the dilution water at test initiation. Hardness and alkalinity measurements will be made by titration using procedures based on methods in *Standard Methods for the Examination of Water and Wastewater* (5). Specific conductance will be measured using a Yellow Springs Instrument Model 33 Salinity-Conductivity-Temperature meter, or equivalent. Additional water quality measurements may be taken as deemed necessary by study personnel. The reason for the additional measurements will be documented in the raw data and summarized in the final report.

Biological Measurements

Observations of mortality and clinical signs of toxicity will be made between 0-24 hours and at 24, 48, 72 and 96 hours \pm 1 hour. Lethality is defined as the lack of visible movement (e.g. lack of fin or opercular movement) in the fish after gentle prodding. All clinical observations including abnormal behavior will be noted.

Sampling for Analytical Measurements

Water samples will be collected from each test chambers at the beginning of the test and at 48 and 96 hours (\pm 1 hour) to determine concentrations of the test substance. In the event that 100% mortality occurs in any treatment, then sampling of that treatment will terminate following the next sampling interval. Samples will be collected at mid-depth from each test chamber and analyzed immediately, or placed in an appropriate storage container (e.g., glass or polypropylene bottle) and stored under refrigeration until analyzed. The sample scheme is summarized below:

PROPOSED NUMBERS OF VERIFICATION SAMPLES

Experimental Group	0 Hour	48 Hours	96 Hours
Control	2	2	2
Solvent Control (if needed)	2	2	2
Level 1- 1.2 ppm	2	2	2
Level 2- 1.8 ppm	2	2	2
Totals	8	8	8

Total Number of Verification Samples = 24

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The above numbers of samples represent those collected from the test and do not include quality control (QC) samples such as matrix blanks and fortifications prepared and analyzed during the analytical chemistry phase of the study. The actual number of samples collected and/or analyzed will be documented in the raw data. At the discretion of the Study Director, water samples from one or more appropriate test chambers and/or stock solutions will be collected and analyzed if an analytical error in sampling or analysis is suspected, or if a malfunction in the test substance delivery system occurs. The reason for the additional samples will be documented in the raw data and summarized in the final report.

Analytical Chemistry

Chemical analysis of the samples will be performed by Wildlife International, Ltd. The analytical method used will be based upon chromatographic methodology provided by the Sponsor and/or developed at Wildlife International, Ltd. The methodology used to analyze the test samples will be documented in the raw data and summarized in the final report.

Data Analysis

When the dose-response pattern allows calculation of an LC50 value, the data will be analyzed using the computer software of C.E. Stephan (6). The program was designed to calculate the LC50 value and the 95% confidence interval by probit analysis, the moving average method, or binomial probability with nonlinear interpolation (7,8,9). The LC50 value will be calculated, when possible, using mortality data collected at 24, 48, 72 and 96 hours. Additional analysis of data may be conducted if deemed appropriate by the Study Director. The results of the analysis will be documented in the raw data and summarized in the final report. The no-mortality concentration and the no-observed-effect concentration (NOEC) will be determined by visually interpreting the clinical observation data.

RECORDS TO BE MAINTAINED

Records to be maintained for data generated by Wildlife International, Ltd. will include, but will not be limited to:

1. A copy of the signed protocol.
2. Identification and characterization of the test substance, if provided by the Sponsor.
3. Dates of initiation and termination of the test.
4. Test organism holding and acclimation records.

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5. Stock solution calculation and preparation, if applicable.
6. Observations.
7. Water chemistry results (e.g., alkalinity and hardness).
8. The methods used to analyze test substance concentrations and the results of analytical measurements, if applicable.
9. Statistical calculations, if applicable.
10. Test conditions (light intensity, photoperiod, etc.).
11. Calculation and preparation of test concentrations.
12. Copy of final report.

FINAL REPORT

A final report of the results of the study will be prepared by Wildlife International, Ltd. The report will include, but not be limited to the following, when applicable:

1. Name and address of the facility performing the study.
2. Dates upon which the study was initiated and completed, and the definitive experimental start and termination dates.
3. A statement of compliance signed by the Study Director addressing any exceptions to Good Laboratory Practice Standards.
4. Objectives and procedures, as stated in the approved protocol, including all changes to the protocol.
5. The test substance identification including name, chemical abstract number or code number, strength, purity, composition, and other information provided by the Sponsor.
6. Stability and solubility of the test substance under the conditions of administration, if provided by the Sponsor.
7. A description of the methods used to conduct the test.
8. A description of the test organisms, including the source, scientific name, age or life stage, lengths and weights of a representative group of test organisms and feed types.
9. A description of the preparation of the test solutions.
10. The methods used to allocate organisms to test chambers and begin the test, the number of organisms and chambers per treatment, the duration of the test and environmental conditions during the test.
11. A description of circumstances that may have affected the quality or integrity of the data.

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Wildlife International, Ltd.

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12. The name of the Study Director and the names of other scientists, professionals, and supervisory personnel involved in the study.
13. A description of the transformations, calculations, and operations performed on the data, a summary and analysis of the biological data and analytical chemistry data, and a statement of the conclusions drawn from the analyses. A graph plotting the concentration-mortality curve at 96 hours. If the data are conducive to evaluation by probit analysis, the slope of the concentration-response curve will be reported.
14. Statistical methods used to evaluate the data, if applicable.
15. The signed and dated reports of each of the individual scientists or other professionals involved in the study.
16. The location where raw data and final report are to be stored.
17. A statement prepared by the Quality Assurance Unit listing the dates that study inspections and audits were made and the dates of any findings reported to the Study Director and Management.
18. If it is necessary to make corrections or additions to a final report after it has been accepted, such changes will be made in the form of an amendment issued by the Study Director. The amendment will clearly identify the part of the final report that is being amended and the reasons for the amendment, and will be signed by the Study Director.

CHANGES TO PROTOCOL

Planned changes to the protocol will be in the form of written amendments signed by the Study Director and approved by the Sponsor's Representative. Amendments will be considered as part of the protocol and will be attached to the final protocol. Any other changes will be in the form of written deviations signed by the Study Director and filed with the raw data. All changes to the protocol will be indicated in the final report.

GOOD LABORATORY PRACTICES

This study will be conducted in accordance with Good Laboratory Practice Standards for EPA (40 CFR Part 160 and/or Part 792); OECD Principles of Good Laboratory Practices (ENV/MC/CHEM (98) 17); and Japan MAFF (11 NohSan, Notification No. 6283, Agricultural Production Bureau, 1 October 1999). Each study conducted by Wildlife International, Ltd. is routinely examined by the Wildlife International, Ltd. Quality Assurance Unit for compliance with Good Laboratory Practices, Standard Operating Procedures and the specified protocol. A statement of compliance with Good Laboratory

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Practices will be prepared for all portions of the study conducted by Wildlife International, Ltd. The Sponsor will be responsible for compliance with Good Laboratory Practices for procedures performed by other laboratories (e.g., residue analyses or pathology). Raw data for all work performed at Wildlife International, Ltd. and a copy of the final report will be filed by project number in archives located on the Wildlife International, Ltd. site or at an alternative location to be specified in the final report.

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Wildlife International, Ltd.

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REFERENCES

- 1 **Organization for Economic Cooperation and Development.** 1993. *Guideline 203: Fish, Acute Toxicity Test.* OECD Guideline for Testing of Chemicals. Updated Guideline adopted on 17 July 1992.
- 2 **U.S. Environmental Protection Agency.** 1996. *Fish Acute Toxicity Test, Freshwater and Marine.* Series 850-Ecological Effects Test Guidelines (*draft*), OPPTS Number 850.1075.
- 3 **ASTM Standard E729-88a.** 1994. *Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians.* American Society for Testing and Materials.
- 4 **Eddy, S.** 1974. *The Freshwater Fishes.* Wm. C. Brown Company Publishers, Dubuque, Iowa.
- 5 **APHA, AWWA, WPCF.** 1998. *Standard Methods for the Examination of Water and Wastewater.* 20th Edition, American Public Health Association. American Water Works Association. Water Pollution Control Federation, New York.
- 6 **Stephan, C.E.** 1978. U.S. EPA, Environmental Research Laboratory, Duluth, Minnesota. Personal communication.
- 7 **Thompson, W.R.** 1947. *Bacteriological Reviews.* Vol. II, No. 2. Pp. 115-145.
- 8 **Stephan, C.E.** 1977. "Methods for Calculating an LC50," *Aquatic Toxicology and Hazard Evaluations.* American Society for Testing and Materials. Publication Number STP 634, pp 65-84.
- 9 **Finney, D.J.** 1971. *Statistical Methods in Biological Assay.* Second edition. Griffin Press, London.

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Wildlife International, Ltd.

Project Number 439A-123

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AMENDMENT TO STUDY PROTOCOL

STUDY TITLE: Tetrabromobisphenol A: A 96-Hour Flow-Through Acute Toxicity Test with the Rainbow Trout (*Oncorhynchus mykiss*)

PROTOCOL NO.: 439/021303/RBT-96H2/OECD-OPPTS/SUB439 **AMENDMENT NO.:** 1

SPONSOR: American Chemistry Council's
Brominated Flame Retardant Industry Panel

PROJECT NO.: 439A-123

EFFECTIVE DATE: July 3, 2003

AMENDMENT: Objective, Page 3:

CHANGE: The objective of this study is to determine the acute effects of Tetrabromobisphenol A (TBBPA) on the rainbow trout (*Oncorhynchus mykiss*) during a 96-hour exposure period under flow-through test conditions.

TO: The objective of this study is to determine if the acute toxicity of Tetrabromobisphenol A (TBBPA) to the rainbow trout, *Oncorhynchus mykiss*, during a 96-hour exposure period under flow-through test conditions would be > 1.0 mg active ingredient (a.i.)/L.

REASON: To add additional wording requested by the sponsor.


STUDY DIRECTOR

7-8-03
DATE


LABORATORY MANAGEMENT

7-8-03
DATE

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Appendix 5

Analytical Report for Tetrabromobisphenol A (TBBPA)

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ALBEMARLE CORPORATION
RESEARCH AND DEVELOPMENT DEPARTMENT

FINAL REPORT ON THE CHEMICAL CHARACTERIZATION (IDENTITY AND PURITY) OF TETRABROMOBISPHENOL-A (TBBPA) IN SUPPORT OF "A 48-HOUR FLOW-THROUGH ACUTE TOXICITY TEST WITH THE CLADOCERAN (*Daphnia magna*) AND A 96-HOUR FLOW-THROUGH ACUTE TOXICITY TEST WITH RAINBOW TROUT (*Oncorhynchus mykiss*)", CONDUCTED BY WILDLIFE INTERNATIONAL, LTD.

- I. Protocol Number: TBBPA-05-29-2003
- II. Sponsor: American Chemistry Council
Brominated Flame Retardant Industry Panel
1300 Wilson Boulevard
Arlington, Virginia 22209
Study Monitor: Wendy K. Sherman
- III. Analytical Testing Facilities: Albemarle Corporation
Process Development Center
Gulf States Road
Baton Rouge, LA 70805
Study Chemist: Paul F. Ranken, Ph. D.
- IV. Date of Study Initiation: June 2, 2003
Date of Study Completion: June 16, 2003
- V. Test Article: A sample of the test article, an end of study sample of Tetrabromobisphenol-A (WIL Test Substance 5754), was analyzed at the Albemarle Process Development Center. WIL Test Substance 5754 is a composite of commercial product from Albemarle Corporation, Great Lakes Chemical Corporation and the Dead Sea Bromine Group. Wildlife International Ltd., Easton, MD 21601, prepared the composite.
- VI. Objective/Methodology: This study was initiated to confirm the identity of the test article and to demonstrate the purity of the test article. Fourier Transform Infrared Spectroscopy using SOP No. ARS-284-R4 confirmed the identity of the test article. In this procedure, the test article infrared spectrum was compared to a standard reference spectrum of TBBPA (Aldrich TBBPA, lot 03120DI). High Performance Liquid Chromatography (HPLC)

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using SOP No. ARS-443-R2 determined the purity (area % TBBPA) of the test article. The test article was further characterized by measuring the concentration (area%) of three potential impurities: tribromophenol, tribromobisphenol-A and o,p'-tetrabromobisphenol-A. Chain of Custody and sample handling were conducted according to established standard operating procedures.

VII. Results:

The Table contains the test article analytical data from the study. Fourier Transform Infrared Spectroscopy confirmed the identity of the test article. High Performance Liquid Chromatography (HPLC) determined the purity of the test article to be 99.27%. Further characterization of the test article was accomplished by measuring the concentration of the three expected impurities. There were no circumstances that may have affected the quality or integrity of the data.

VIII. Regulatory Requirements:

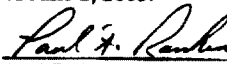
The study conformed to the requirements of EPA TSCA (40 CFR Part 792) Good Laboratory Practice Regulations and the OECD [C(97)186/Final] Good Laboratory Practice Regulations.

IX. Data/Record Retention:

All log books, spectra and reports will be forwarded to the Quality Assurance Unit (QAU) for a final review prior to filing in the designated Health and Environment archives at Albemarle Corporation, Health and Environment Department, 451 Florida Street, Baton Rouge, LA 70801.

X. Protocol Amendment:

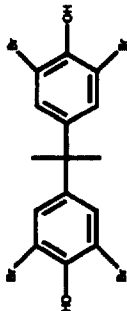
The protocol was amended on June 12, 2003 to reflect a change in the approval date. The approval date originally listed by the study chemist was incorrectly listed as June 30, 2003. The amendment shows the correct approval date of June 2, 2003.


Paul F. Ranken, Ph.D.
STUDY CHEMIST

June 16, 2003
DATE

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Table 1. CONCLUSIONS AND TEST ARTICLE ANALYTICAL DATA FOR TBBPA, WIL Test Substances #5754

CHEMICAL NAME: Tetrabromobisphenol-A					
C.A.S. No.: 79-94-7					
MOLECULAR FORMULA: C ₁₅ H ₁₂ Br ₄ O ₂					
PHYSICAL FORM: White Powder					
CHEMICAL STRUCTURE:					
					
ANALYSIS		RESULTS		ANALYSIS DATES	ANALYST
		Area %			
	TBBPA	2,4,6-Tribromophenol	o,p-tetrabromobisphenol-A	Tribromobisphenol-A	
HPLC	99.27	0.01	0.04	0.68	
FT-IR	The sample FT-IR spectrum matched that of the Aldrich reference spectrum. All spectra are on file with the original data.				
CONCLUSION: Based on these analytical data, the test article identity was confirmed as tetrabromobisphenol-A. The test article was shown to have a purity of 99.27%.					

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**CHEMICAL CHARACTERIZATION ANALYTICAL PROTOCOL
FOR DETERMINING THE IDENTITY AND PURITY
OF TETRABROMOBISPHENOL-A (TBBPA) IN SUPPORT OF "A 48-HOUR
FLOW-THROUGH ACUTE TOXICITY TEST WITH THE CLADOCERAN (*Daphnia
magna*) AND A 96-HOUR FLOW-THROUGH ACUTE TOXICITY TEST WITH
RAINBOW TROUT (*Oncorhynchus mykiss*)", CONDUCTED BY WILDLIFE
INTERNATIONAL, LTD.**

PROTOCOL AMENDMENT

Date: June 16, 2003

Section to be changed: Section X, page 2

Change: Date of protocol approval is June 2, 2003.

Reason for change: The protocol approval date originally listed by the Study Chemist was incorrectly given as June 30, 2003. The amendment shows the correct approval date of June 2, 2003.

June 16, 2003
Date:

Paul F. Ranken
Paul F. Ranken
Study Chemist

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Appendix 6

Personnel Involved in the Study

The following key Wildlife International, Ltd. personnel were involved in the conduct or management of this study:

1. Henry O. Krueger, Ph.D., Director of Aquatic Toxicology/Terrestrial Plants and Insects
2. Willard B. Nixon, Ph.D., Director of Chemistry
3. Cary A. Sutherland, Laboratory Supervisor
4. Raymond L. Van Hoven, Scientist
5. Frank J. Lezotte, Chemist
6. Amy S. Blankinship, Aquatic Biologist

HEXABROMOCYCLODODECANE (HBCD): AN ACTIVATED SLUDGE,
RESPIRATION INHIBITION TEST

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 439E-108A

Organisation for Economic Cooperation and Development
OECD Guideline 209

and

Council of European Communities Directive 67/548/EEC
Annex V, Guideline C.11

AUTHORS:
Edward C. Schaefer
Abul I. Siddiqui

STUDY INITIATION DATE: June 19, 2003

STUDY COMPLETION DATE: August 20, 2003

SUBMITTED TO:

American Chemistry Council's
Brominated Flame Retardant Industry Panel
1300 Wilson Boulevard
Arlington, Virginia 22209

Wildlife International, Ltd.

8598 Commerce Drive
Easton, Maryland 21601
(410) 822-8600

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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

SPONSOR: American Chemistry Council's Brominated Flame Retardant Industry Panel

TITLE: HEXABROMOCYCLODODECANE (HBCD): An Activated Sludge, Respiration Inhibition Test

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 439E-108A

STUDY COMPLETION: August 20, 2003

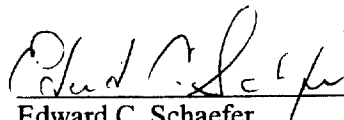
This study was conducted in compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency in EPA 40 CFR Part 160, 17 August 1989; OECD Principles of Good Laboratory Practices (ENV/MC/CHEM (98) 17), and Japan MAFF (11 NohSan, Notification No. 6283, Agricultural Production Bureau, 1 October 1999), with the following exceptions:

The reference substance, obtained from Aldrich Chemical Company (Milwaukee, WI), was not characterized in compliance with Good Laboratory Practice Standards.

The stability of the reference substance under conditions of storage at the test site was not determined in accordance with Good Laboratory Practice Standards.

The homogeneity and stability of the reference substance in the carrier was not determined in accordance with Good Laboratory Practice Standards.

STUDY DIRECTOR:



Edward C. Schaefer
Manager, Biodegradation

20 August 2003
DATE

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QUALITY ASSURANCE STATEMENT

This study was examined for compliance with Good Laboratory Practice as published by the U.S. Environmental Protection Agency in EPA 40 CFR Part 160, 17 August 1989; OECD Principles of Good Laboratory Practices (ENV/MC/CHEM (98) 17); and Japan MAFF (11 NohSan, Notification No, 6283, Agricultural Production Bureau 1 October 1999); Wildlife International, Ltd. Standard Operating Procedures and the study protocol. The dates of all inspections and audits and the dates that any findings were reported to the Study Director and Laboratory Management were as follows:

ACTIVITY:	DATE CONDUCTED:	DATE REPORTED TO:	
		STUDY DIRECTOR:	MANAGEMENT
<u>Initial Trial: 439E-108</u>			
Test Substance Preparation, Test Initiation	June 24, 2003	June 24, 2003	June 26, 2003
D. O. Measurements	June 24, 2003	June 24, 2003	June 27, 2003
Protocol	June 25, 2003	June 25, 2003	June 26, 2003
<u>Definitive Trial: 439E-108A</u>			
D. O. Measurements	June 25, 2003	June 25, 2003	June 27, 2003
Data & Draft Report	July 15 & 16, 2003	July 16, 2003	July 21, 2003
Final Report	August 18, 2003	August 18, 2003	August 20, 2003

All inspections were study-based unless otherwise noted.



Robert N. McGee, B.S.
Quality Assurance Representative

August 20, 2003
DATE

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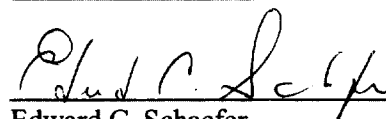
REPORT APPROVAL

SPONSOR: American Chemistry Council's Brominated Flame Retardant Industry Panel

TITLE: HEXABROMOCYCLODODECANE (HBCD): An Activated Sludge, Respiration Inhibition Test

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 439E-108A

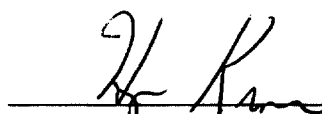
STUDY DIRECTOR:



Edward C. Schaefer
Manager, Biodegradation

20 August 2003
DATE

MANAGEMENT:



Henry O. Krueger, Ph.D.
Director, Aquatic Toxicology and Non-Target Plants

20 Aug 03
DATE

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STUDY INFORMATION

Study Initiation Date: June 19, 2003
Experimental Start Date: June 23, 2003
Experimental Termination Date: June 25, 2003
Study Completion Date: August 20, 2003

Study Director: Edward C. Schaefer

Sponsor: American Chemistry Council's
Brominated Flame Retardant Industry Panel
1300 Wilson Boulevard
Arlington, Virginia 22209

Sponsor's Representative: Ms. Wendy Sherman

Study Personnel: Edward C. Schaefer, B.S., Manager, Biodegradation
Henry O. Krueger, Ph.D., Director, Aquatic Toxicology and
Non-Target Plants
Abul Siddiqui, B.A., Scientist, Biodegradation

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ABSTRACT

The effect of Hexabromocyclododecane (HBCD) on activated sludge microorganisms was assessed by the Activated Sludge Respiration Inhibition Test Method (OECD Guideline 209). The test contained control, reference substance and treatment groups. The control group was used to determine the background respiration rate of the sludge and was not dosed with the test or reference substance. The reference group was dosed with 3,5-dichlorophenol, a known inhibitor of respiration, at nominal concentrations of 3, 15 and 50 mg/L. The test substance was dosed at a limit concentration of 15 mg/L. After an exposure period of approximately three hours, the respiration rates of the test solutions were measured using a dissolved oxygen meter. The individual respiration rates of the two controls were 60.5 and 55.5 mg O₂/L/hr. The difference between the two control respiration rates was 9.0% and was within the 15% difference limit established for the test. The validity of the test was further supported by the results from the 3,5-dichlorophenol reference group, which resulted in an EC₅₀ of 5.2 mg/L. The EC₅₀ was within the 5 to 30 mg/L range considered acceptable for the test. An average of approximately 29.1 percent inhibition was observed in the treatment group. Following is a summary of the results:

Treatment/Nominal Concentration	Respiration Rate mg O ₂ /L/hour	Percent Inhibition
Control 1	60.5	NA
Control 2	55.5	NA
3,5-dichlorophenol 3 mg/L	38.9	32.9
3,5-dichlorophenol 15 mg/L	11.0	81.1
3,5-dichlorophenol 50 mg/L	0.0	100.0
Hexabromocyclododecane (HBCD) 15 mg/L	42.4	26.9
Hexabromocyclododecane (HBCD) 15 mg/L	41.0	29.3
Hexabromocyclododecane (HBCD) 15 mg/L	40.0	31.0

NA – Not applicable

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INTRODUCTION

The purpose of this test is to provide a screening method to identify substances that may adversely affect aerobic microbial treatment plants and to indicate suitable non-inhibitory test substance concentrations for use in biodegradability tests.

This study was conducted by Wildlife International, Ltd. for the American Chemistry Council's Brominated Flame Retardant Industry Panel at the Wildlife International, Ltd. biodegradation facility in Easton, Maryland. Original raw data generated by Wildlife International, Ltd. and the original final report are filed under Project Number 439E-108A in the archives located on the Wildlife International, Ltd. site.

OBJECTIVE

The objective of this study was to assess the effects of Hexabromocyclododecane (HBCD) on activated sludge microorganisms by measuring the respiration rate.

EXPERIMENTAL DESIGN

The test contained control, reference substance, and treatment groups. The control group was used to determine the background respiration rate of the sludge and was not exposed to the test or reference substances. The reference group was dosed with 3,5-dichlorophenol, a known inhibitor of respiration, at nominal concentrations of 3, 15 and 50 mg/L. The test substance was tested at a limit concentration of 15 mg/L, in triplicate.

MATERIALS AND METHODS

This study was conducted according to the procedures outlined in the protocol, "HEXABROMOCYCLODODECANE (HBCD): AN ACTIVATED SLUDGE, RESPIRATION INHIBITION TEST" (Appendix II). The protocol was based on the procedures specified in the OECD Guideline for Testing of Chemicals, Method 209 (1) and Council of the European Communities, Guideline C.11, Activated Sludge, Respiration Inhibition Test (2).

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Test Substance

The test substance used in this study was a composite of the following three samples:

Manufacturer:	Albemarle
Sample ID:	SAYTEX HBCD-LM 0.6 #
Description	White Powder
Purity	Not given
Lot No.:	33449-15X
CAS No:	Not given
Expiration Date:	June 01, 2001
Date Received:	December 20, 1995
Wildlife International, Ltd. ID:	3519
Manufacturer:	Great Lakes Chemical Corporation
Sample ID:	Hexabromocyclododecane
Description	White powder
Purity	Not given
Batch No.:	Not given
CAS No:	3194-55-6
Expiration Date:	Not given
Date Received:	June 19, 1998
Wildlife International, Ltd. ID:	4515
Manufacturer:	Eurobrom B.V
Sample ID:	HBCD
Description	White powder
Purity	99.4% (Certificate of Analysis)
Lot No.:	010328
CAS No:	25637-99-4
Expiration Date:	Not given
Date Received:	December 10, 2001
Wildlife International, Ltd. ID:	5827

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The composite test substance was assigned Wildlife International, Ltd. identification number 5850 and was stored under ambient conditions. The composite test substance was shipped to Albemarle Corporation for characterization and purity analyses. The results of the analyses indicated the composite test substance was homogeneous, and the test substance was assigned an expiration date of February 21, 2003. Since this test was conducted after the expiration date, another sample of the composite test substance was shipped to Albemarle Corporation to extend the expiration date. The results of the analyses indicated the composite test substance had a purity of 95.86% and a new expiration date of June 27, 2004, one year after the date of analysis (Appendix III).

The test substance was administered to the treatment group by direct weight addition.

Reference Substance

A stock solution of the reference substance, 3,5-dichlorophenol was prepared by dissolving 500 mg in 10 mL of 1N NaOH and then diluting to 30 mL with NANOpure[®] water. While stirring, enough 1N H₂SO₄ was added to reach the point of incipient precipitation. The solution of 3,5-dichlorophenol then was diluted to 1 L with NANOpure[®] water. The reference substance was administered by volumetric addition. Following is a description of the reference substance used in this study.

Name:	3,5-dichlorophenol
Manufacturer:	Aldrich Chemical Co., Milwaukee, WI
Lot Number:	02611ES
Physical Description:	White solid
Handling Precautions:	Standard laboratory precautions
Date Received:	January 24, 2000
Expiration Date:	January 24, 2005
Purity:	99.1%
Storage Conditions:	Ambient
CAS Number:	591-35-5
Wildlife International, Ltd. ID:	5179

Test Conditions and Apparatus

Control, reference, and treatment test mixtures were incubated at $20 \pm 2^{\circ}\text{C}$ and aerated for three hours at a rate sufficient to provide aerobic conditions and maintain solids in suspension. The mixtures

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were prepared and aerated in 500 mL plastic Erlenmeyer flasks and then transferred into 300 mL biochemical oxygen demand (BOD) bottles to conduct the dissolved oxygen (DO) measurements.

Test Inoculum

Activated sludge was collected from the Denton Wastewater Treatment Plant, Denton, Maryland on June 23, 2003. The Denton facility receives wastes from predominately domestic sources. The sludge was sieved using a 2 mm screen and allowed to settle for approximately 30 minutes. After the settling period, the supernatant was removed and the total suspended solids (TSS) concentration of the settled sludge was determined.

The sludge was maintained in the laboratory for 2 days prior to use. Approximately 50 mL of synthetic sewage (Protocol, Appendix II) was added to each liter of activated sludge and the sludge was continuously aerated. Before use, the pH and total suspended solids concentration of the activated sludge were determined.

Procedure

Test mixtures were prepared at 15 minute intervals starting with the first control. The control contained 9.6 mL of synthetic sewage, 120 mL of inoculum, and enough municipal water to bring the total volume up to 300 mL. The mixture was promptly aerated at a rate sufficient to provide aerobic conditions and keep the solids in suspension. Subsequent mixtures contained 9.6 mL of synthetic sewage, 120 mL of inoculum, the appropriate amount of test substance or reference substance stock solution, and enough municipal water to bring the total volume up to 300 mL. Finally, a second control was prepared. All mixtures were aerated for three hours.

Sample Analysis

After three hours of aeration, the contents of the first vessel were transferred to a BOD bottle and the respiration rate was measured over a period of up to 10 minutes. Dissolved oxygen readings were recorded every 10 seconds for 10 minutes or until the DO dropped below 1.0 mg/L, whichever came first using a YSI Model 50B Dissolved Oxygen Meter. The respiration rate in subsequent vessels was determined in an identical manner at 15 minute intervals so that the contact time of the test substance with the activated sludge was three hours.

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Calculations

A respiration rate was calculated for each test mixture and expressed in mg O₂/L/hour. The rate was calculated using DO values between approximately 6.5 mg O₂/L and 2.5 mg O₂/L, or over a 10 minute period if the DO did not reach approximately 2.5 mg O₂/L. The respiration rate was calculated using the following equation:

$$\text{Respiration Rate} = (\text{initial DO} - \text{final DO}) / (\text{final time} - \text{initial time})$$

Percent inhibition was calculated using the following equation:

$$\text{Percent Inhibition} = 1 - \frac{2R_s}{RC_1 + RC_2} \times 100$$

where:

- R_s = oxygen consumption rate at a given concentration of the test substance
- RC₁ = oxygen consumption rate, Control 1
- RC₂ = oxygen consumption rate, Control 2

Statistical Analyses

When the dose response pattern allows for the calculation of an EC50 value, the data are analyzed using the computer program of C.E. Stephan (3). The program was designed to calculate the EC50 value and the 95% confidence interval by probit analysis, the moving average, or binomial probability with nonlinear interpolation (4, 5, 6). The EC50 value for the reference group was calculated using nonlinear interpolation.

RESULTS AND DISCUSSION

The temperature range during the maintenance of the sludge and during the test was 20-21° C. The measured total suspended solids (TSS) concentration and pH of the sludge on the day of testing was 4213 mg/L and 7.8, respectively.

Respiration rates and percent inhibitions are presented in Table 1. The respiration rates in the two controls were 60.5 and 55.5 mg O₂/L/hr. The difference between the two control respiration rates was 9.0% and was within the 15% difference limit established for the test. The validity of the test was further

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supported by the results from the 3,5-dichlorophenol reference group, which resulted in an EC50 of 5.2 mg/L. The EC50 was within the 5 to 30 mg/L range considered acceptable for the test.

Inhibitory effects upon respiration were observed at a Hexabromocyclododecane (HBCD) concentration of 15 mg/L. The average respiration rate for the treatment group was 41.1 ± 1.2 O₂/L/hr and was lower than the average respiration rate of the control (58.0 ± 3.5 mg O₂/L/hr). The average percent inhibition observed was approximately $29.1\% \pm 2.1$ mg O₂/L/hr.

CONCLUSION

Minimal inhibitory effects upon respiration were observed at a Hexabromocyclododecane (HBCD) concentration of 15 mg/L. The average percent inhibition observed was approximately 29.1%.

REFERENCES

1. **Organisation for Economic Cooperation and Development.** 1989. *Activated Sludge Respiration Inhibition Test*. OECD Guideline 209.
2. **Council of the European Communities.** Directive 67/548/EEC. Annex V. Guideline C.11, *Activated Sludge Respiration Inhibition Test*.
3. **Stephan, C.E.** 1977. "Methods for Calculating an LC50," *Aquatic Toxicology and Hazard Evaluations*. American Society for Testing and Materials. Publication Number STP 634, pp 65-84.
4. **Finney, D.J.** 1971. *Statistical Methods in Biological Assay*, second edition. Griffin Press, London.
5. **Thompson, W.R.** 1947. *Bacteriological Reviews*, Vol. II, No. 2: 115-145.
6. **Stephan, C.E.** 1977. "Methods for Calculating an LC50," *Aquatic Toxicology and Hazard Evaluations*. American Society for Testing and Materials. Publication Number STP 634, pp 65-84.

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Table 1

Respiration Rates and Percent Inhibitions

Treatment/Nominal Concentration	Respiration Rate mg O ₂ /L/hour	Percent Inhibition
Control 1	60.5	NA
Control 2	55.5	NA
3,5-dichlorophenol 3 mg/L	38.9	32.9
3,5-dichlorophenol 15 mg/L	11.0	81.1
3,5-dichlorophenol 50 mg/L	0.0	100.0
Hexabromocyclododecane (HBCD) 15 mg/L	42.4	26.9
Hexabromocyclododecane (HBCD) 15 mg/L	41.0	29.3
Hexabromocyclododecane (HBCD) 15 mg/L	40.0	31.0
NA – Not applicable.		

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Appendix I

Measured Dissolved Oxygen (DO) Concentrations (mg O₂/L)

Time (min./sec.)	Control 1	Reference			Hexabromocyclododecane (HBCD)			Control 2
		3 mg/L	15 mg/L	50 mg/L	Rep A 15 mg/L	Rep B 15 mg/L	Rep C 15 mg/L	
00:10	6.9	7.8	8.7	8.7	7.7	7.6	6.8	6.8
00:20	6.2	7.6	8.7	9.1	7.4	7.2	6.5	6.2
00:30	5.8	7.4	8.6	9.2	7.2	7.0	6.3	5.8
00:40	5.5	7.2	8.6	9.2	7.0	6.7	6.1	5.6
00:50	5.3	7.1	8.6	9.2	6.9	6.6	5.9	5.4
00:60	5.1	6.9	8.5	9.2	6.7	6.4	5.8	5.2
00:70	4.9	6.8	8.5	9.3	6.6	6.3	5.7	5.1
00:80	4.7	6.7	8.5	9.3	6.5	6.2	5.6	4.9
00:90	4.6	6.6	8.4	9.3	6.3	6.1	5.5	4.7
01:00	4.4	6.5	8.4	9.3	6.2	5.9	5.4	4.6
01:10	4.3	6.4	8.4	9.3	6.1	5.8	5.3	4.5
01:20	4.1	6.3	8.3	9.3	6.0	5.7	5.2	4.3
01:30	4.0	6.2	8.3	9.2	5.9	5.6	5.1	4.2
01:40	3.9	6.0	8.3	9.2	5.7	5.5	5.0	4.1
01:50	3.7	5.9	8.3	9.2	5.6	5.3	4.9	3.9
01:60	3.6	5.8	8.2	9.2	5.5	5.2	4.8	3.8
01:70	3.4	5.7	8.2	9.2	5.4	5.1	4.6	3.7
01:80	3.3	5.6	8.2	9.2	5.3	5.0	4.6	3.6
01:90	3.2	5.5	8.1	9.2	5.2	4.9	4.5	3.4
02:00	3.0	5.4	8.1	9.1	5.1	4.8	4.3	3.3
02:10	2.9	5.3	8.1	9.1	4.9	4.7	4.2	3.2
02:20	2.7	5.2	8.1	9.1	4.8	4.6	4.1	3.0
02:30	2.6	5.1	8.0	9.1	4.7	4.5	4.0	2.9
02:40	2.5	5.0	8.0	9.1	4.6	4.4	3.9	2.8
02:50	2.4	4.9	8.0	9.1	4.5	4.2	3.8	2.6
02:60	2.2	5.8	7.9	9.1	4.4	4.1	3.7	2.5
02:70	2.1	4.7	7.9	9.1	4.3	4.0	3.6	2.3
02:80	2.0	4.6	7.9	9.1	4.1	3.9	3.5	2.2
02:90	1.8	4.8	7.9	9.0	4.0	3.8	3.4	2.0
03:00	1.7	4.4	7.8	9.0	3.9	3.7	3.3	1.9
03:10	1.6	4.2	7.8	9.0	3.8	3.6	3.2	1.8
03:20	1.4	4.1	7.8	9.0	3.7	3.5	3.1	1.6
03:30	1.3	4.0	7.7	9.0	3.6	3.4	3.0	1.5
03:40	1.2	3.9	7.7	9.0	3.4	3.3	2.9	1.4
03:50	1.0	3.8	7.7	9.0	3.3	3.2	2.8	1.3
03:60	0.9	3.7	7.6	9.0	3.2	3.1	2.7	1.1
03:70		3.6	7.6	9.0	3.1	2.9	2.6	1.0
03:80		3.5	7.6	9.0	3.0	2.8	2.5	0.9
03:90		3.4	7.5	9.0	2.9	2.7	2.4	
04:00		3.3	7.5	8.9	2.7	2.6	2.3	
04:10		3.2	7.5	8.9	2.6	2.5	2.2	
04:20		3.1	7.4	8.9	2.5	2.4	2.1	
04:30		2.9	7.4	8.9	2.4	2.3	2.0	
04:40		2.8	7.4	8.9	2.3	2.2	1.9	
04:50		2.7	7.3	8.9	2.2	2.1	1.8	
04:60		2.6	7.3	8.9	2.0	2.0	1.7	
04:70		2.5	7.3	8.9	1.9	1.9	1.6	
04:80		2.4	7.3	8.8	1.8	1.8	1.5	
04:90		2.3	7.2	8.8	1.7	1.7	1.4	
05:00		2.2	7.2	8.8	1.5	1.5	1.3	
05:10		2.0	7.2	8.8	1.4	1.4	1.2	
05:20		1.9	7.1	8.8	1.2	1.3	1.1	
05:30		1.8	7.1	8.8	1.1	1.2	1.0	
05:40		1.7	7.1	8.8	1.0	1.1	0.9	
05:50		1.6	7.0	8.8	0.9	1.0		
05:60		1.5	7.0	8.8		0.9		
05:70		1.4	7.0	8.8				
05:80		1.3	6.9	8.8				
05:90		1.2	6.9	8.7				
06:00		1.0	6.9	8.7				

Bold numbers indicate dissolved oxygen concentrations used to calculate respiration rates.

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Appendix II

Protocol

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PROTOCOL

HEXABROMOCYCLODODECANE (HBCD): AN ACTIVATED
SLUDGE, RESPIRATION INHIBITION TEST

Organization for Economic Cooperation and Development
OECD Guideline 209

and

Council of European Communities Directive 67/548/EEC
Annex V, Guideline C.11

Submitted to

American Chemistry Council's
Brominated Flame Retardant Industry Panel
1300 Wilson Boulevard
Arlington, Virginia 22209

Wildlife International, Ltd.

8598 Commerce Drive
Easton, Maryland 21601
(410) 822-8600

April 15, 2003

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Wildlife International, Ltd.

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HEXABROMOCYCLODODECANE (HBCD): AN ACTIVATED
SLUDGE, RESPIRATION INHIBITION TEST

SPONSOR: American Chemistry Council's
Brominated Flame Retardant Industry Panel
1300 Wilson Boulevard
Arlington, Virginia 22209

SPONSOR'S REPRESENTATIVE: Ms. Wendy Sherman

TESTING FACILITY: Wildlife International, Ltd.
8598 Commerce Drive
Easton, Maryland 21601

STUDY DIRECTOR: Edward C. Schaefer
Wildlife International, Ltd.

LABORATORY MANAGEMENT: Henry O. Krueger, Ph.D.
Director of Aquatic Toxicology/Terrestrial Plants & Insects

FOR LABORATORY USE ONLY

Proposed Dates:	
Experimental Start Date: <u>23 JUNE 2003</u>	Experimental Termination Date: <u>27 JUNE 2003</u>
Project No.: <u>439E-108</u>	
Test Concentrations: <u>15 mg/L</u>	
Test Substance No.: <u>5850</u> Reference Substance No. (if applicable): <u>5179</u>	

PROTOCOL APPROVAL

Edward C. Schaefer
STUDY DIRECTOR

19 JUNE 2003
DATE

H. O. Krueger
LABORATORY MANAGEMENT

19 June 03
DATE

Wendy K. Sherman
SPONSOR'S REPRESENTATIVE

6/5/03
DATE

PROTOCOL NO.: 439/041503/ASRIT/SUB439

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Wildlife International, Ltd.

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INTRODUCTION

The purpose of this test is to provide a screening method to identify substances that may adversely affect aerobic microbial treatment plants and to indicate suitable non-inhibitory test substance concentrations for use in biodegradability tests.

OBJECTIVE

The objective of the study will be to assess the effects of Hexabromocyclododecane (HBCD) on activated sludge microorganisms by measuring the respiration rate. An EC50 will be calculated, if possible.

EXPERIMENTAL DESIGN

The test will contain control, reference, and treatment groups. The control group is used to determine the background respiration rate of the sludge and will not be exposed to the test substance. The reference group will be dosed with 3,5-dichlorophenol, a known inhibitor of respiration, at concentrations of 3, 15, and 50 mg/l. The test substance will be tested at a limit concentration of 15 mg/l, in triplicate.

MATERIALS AND METHODS

Test methods are based on the procedures specified in the OECD Guideline for Testing of Chemicals, Method 209 (1) and Council of the European Communities, Guideline C.11, Activated Sludge, Respiration Inhibition Test (2).

Test Substance

The test substance consisted of a composite of HBCD samples received from three manufacturers. The material's identity and date received from each of the manufacturers is given below:

<u>Manufacturer</u>	<u>Lot/Batch</u>	<u>Date Received</u>	<u>Wildlife International Ltd. Identification Number</u>
Great Lakes Chemical Corporation	Not Given	June 19, 1998	4515
Eurobrom b.v.	010328	December 10, 2001	5827
Albemarle Corporation	33449-15X	December 20, 1995	3519

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Wildlife International, Ltd.

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The composite test substance was assigned Wildlife International Ltd. identification number 5850 and was stored under ambient conditions. A subsample of the composite test substance has been shipped to Albemarle Corporation for characterization and purity analyses. A copy of Albemarle Corporation's final report on characterization and purity determination will be included as an appendix to the results of these analyses.

The test substance will be administered by direct weight addition. Direct weight addition is the most appropriate route of administration of minimally soluble materials.

Stock Solution Preparation

A stock solution of 3,5-dichlorophenol will be prepared by dissolving 500 mg in 10 mL of 1N NaOH and then diluting to 30 mL with NANO™ pure water. While stirring, enough 1N H₂SO₄ (approximately 8 mL) will be added to reach the point of incipient precipitation. The solution of 3,5-dichlorophenol then will be diluted to 1 L with NANO™ pure water. The reference substance will be administered by volumetric addition.

Test System

The biological test system is a consortium of microorganisms common to the activated sludge treatment process. The organisms responsible for the decomposition of organic materials are principally aerobic, and facultative bacteria. The test system was chosen because it is representative of a treatment process that may receive the test substance.

Test Conditions and Apparatus

Control, reference, and treatment test mixtures will be incubated at $20 \pm 2^{\circ}\text{C}$ and aerated for 3 hours at a rate sufficient to maintain solids in suspension. The mixtures will be prepared and aerated in 500 mL plastic Erlenmeyer flasks and then transferred into a 300 mL Biochemical Oxygen Demand (BOD) bottle to conduct dissolved oxygen (DO) measurements. Test mixtures will be identified by project number, test substance identification and test concentration.

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Test Inoculum

Activated sludge from the Denton Wastewater Treatment Plant, Denton, Maryland will be used as the inoculum for the test. The sludge will be sieved using a 2 mm screen and then allowed to settle for approximately 30 minutes. The supernatant above the settled solids will be drained and the total suspended solids (TSS) concentration of the settled sludge will be determined. Based on the result, the concentration of the sludge will be adjusted to 4000 mg/L ($\pm 10\%$) by diluting with Nanopure® water.

If the sludge cannot be used on the day of collection or if the same batch is required to be used on subsequent days (maximum four days), 50 mL of synthetic sewage (Appendix I) will be added to each liter of activated sludge at the end of each working day. The sludge will be aerated overnight at $20 \pm 2^\circ\text{C}$. Before use, the pH and total suspended solids concentration of the activated sludge will be determined and, if necessary, adjusted to pH 6.0 - 8.0 and a solids concentration of 4000 mg/L ($\pm 10\%$).

Procedure

Test mixtures will be prepared at 15 minute intervals starting with the first control. The control will contain 9.6 mL of synthetic sewage, 120 mL of inoculum and enough municipal water to bring the total volume up to 300 mL. The mixture will be promptly aerated at a rate sufficient to keep the solids in suspension. Subsequent mixtures will contain 9.6 mL of synthetic sewage, 120 mL of inoculum, the appropriate amount of test or reference substance, and enough municipal water to bring the total volume up to 300 mL. Finally, a second control will be prepared. All mixtures will be aerated for three hours.

Sample Analysis

After three hours of aeration, the contents of the first vessel will be transferred to a BOD bottle and the respiration rate will be measured over a period of up to 10 minutes. Dissolved oxygen readings will be recorded every 10 seconds for 10 minutes or until the DO drops below 1.0 mg/L, whichever occurs first. The respiration rate in subsequent vessels will be determined in an identical manner at 15 minute intervals so that the contact time of the test substance with the activated sludge is three hours.

Calculations

A respiration rate will be calculated for each test mixture and expressed in mg O₂/L/hour. The rate will be calculated using DO values between approximately 6.5 mg O₂/L and 2.5 mg O₂/L, or over a 10

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minute period if the DO does not reach approximately 2.5 mg O₂/L. The respiration rate will be calculated as follows:

$$\text{Respiration Rate} = (\text{initial DO} - \text{final DO}) / (\text{final time} - \text{initial time})$$

The percent inhibition for each test substance concentration will be calculated using the following equation and plotted against concentration on log paper:

$$\text{Percent Inhibition} = 1 - \frac{2R_s}{RC_1 + RC_2} \times 100$$

where

R_s = oxygen consumption rate at a given concentration of the test substance

RC₁ = oxygen consumption rate, Control 1

RC₂ = oxygen consumption rate, Control 2

An EC50 value will be derived, if possible, based on the percent inhibition versus test substance concentration. Confidence limits (95%) for the EC50 will be determined using standard statistical procedures (3).

Quality Control

The test is considered valid only if the following criteria are met:

- the two control respiration rates are within 15% of each other;
- the EC50 (3 hours) of 3,5-dichlorophenol is in the accepted range of 5 to 30 mg/L.

RECORDS TO BE MAINTAINED

Records to be maintained will include, but not limited to, the following:

1. A copy of the signed protocol.
2. Identification and characterization of the test substance as provided by Sponsor.
3. Test initiation and termination dates.
4. Experimental initiation and termination dates.

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5. Stock solution concentration calculations and solution preparation.
6. Activated sludge source and pretreatment details.
7. Test temperature and duration.
8. Reference substance results.
9. All dissolved oxygen measurements.
10. Temperature range recorded during test period.
11. Inhibition curve and method for calculation of EC50.
12. If calculated, EC50 and 95% confidence limits.
13. A copy of the final report.

FINAL REPORT

A final report of the results of the study will be prepared by Wildlife International, Ltd. The report is to include, but is not limited to, the following when applicable:

1. Name and address of facility performing the study.
2. Dates on which the study was initiated and completed.
3. A statement of compliance signed by the Study Director addressing any exceptions to Good Laboratory Practice Standards.
4. Objectives and procedures stated in the approved protocol, including any changes in the original protocol.
5. Identification and characterization of the test substance as provided by Sponsor including name, CAS number, percent active, and other characteristics, if provided by the Sponsor.
6. A description of the transformations and calculations performed on the data.
7. A description of the methods used and reference to any standard method employed.
8. A description of the test system.
9. A description of the preparation of the test solutions, the testing concentration(s), the route of administration, and the duration of the test.
10. A description of all circumstances that may have affected the quality or integrity of the data.
11. The name of the study director, the names of other scientists or professionals, and the names of all supervisory personnel, involved in the study.
12. The signed and dated reports of each of the individual scientists or other professionals involved in the study, if applicable.

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Wildlife International, Ltd.

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13. The location where the raw data and final report are to be stored.
14. A statement prepared by the Quality Assurance Unit listing the dates that the study inspections and audits were made and the dates of any findings were reported to the Study Director and Management.
15. If it is necessary to make corrections or additions to a final report after it has been accepted, such changes will be made in the form of an amendment issued by the Study Director. The amendment will clearly identify the part of the final report that is being amended and the reasons for the amendment, and will be signed by the Study Director.
16. A copy of the signed protocol and amendments.

CHANGING OF PROTOCOL

Planned changes to the protocol will be in the form of written amendments signed by the Study Director and approved by the Sponsor's Representative. Amendments will be considered as part of the protocol and will be attached to the final protocol. Any other changes will be in the form of written deviations signed by the Study Director and filed with the raw data. All changes to the protocol will be indicated in the final report.

GOOD LABORATORY PRACTICES

This study will be conducted in accordance with Good Laboratory Practice Standards for EPA (40 CFR Part 160); OECD Principles of Good Laboratory Practices (ENV/MC/CHEM (98) 17); and Japan MAFF (59 NohSan, Notification No. 3850, Agricultural Production Bureau). Each study conducted by Wildlife International, Ltd. is routinely examined by the Wildlife International, Ltd. Quality Assurance Unit for compliance with Good Laboratory Practices, Standard Operating Procedures and the specified protocol. A statement of compliance with Good Laboratory Practices will be prepared for all portions of the study conducted by Wildlife International, Ltd. Raw data for all work performed at Wildlife International, Ltd. and a copy of the final report will be filed by project number in archives located on the Wildlife International, Ltd. site, or at an alternative location to be specified in the final report.

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Wildlife International, Ltd.

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REFERENCES

- 1 **Organisation for Economic Cooperation and Development.** 1989. *Activated Sludge Respiration Inhibition Test.* OECD Guideline 209.
- 2 **Council of the European Communities.** Directive 67/548/EEC. Annex V. Guideline C.11, *Activated Sludge Respiration Inhibition Test.*
- 3 **Stephan, C.E.** 1977. "Methods for Calculating an LC50," *Aquatic Toxicology and Hazard Evaluations.* American Society for Testing and Materials. Publication Number STP 634, pp 65-84.

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APPENDIX I. SYNTHETIC SEWAGE

The synthetic sewage provides the necessary nutrients required for bacterial metabolism. It is prepared by dissolving the following amounts of substances in 1 liter of municipal water:

16.0 g peptone
11.0 g meat extract
3.0 g urea
0.7 g NaCl
0.4 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$
0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
2.8 g K_2HPO_4

Reagent grade chemicals or better will be used when available. The constituents of the synthetic sewage are not known to contain any contaminants that are reasonable expected to be present and are known to be capable of interfering with the study.

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Appendix III

Final Report on the Chemical Characterization of Hexabromocyclododecane

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288 7/30/03
CERTIFIED TRUE COPY

**ALBEMARLE CORPORATION
RESEARCH AND DEVELOPMENT DEPARTMENT**

FINAL REPORT ON THE CHEMICAL CHARACTERIZATION (IDENTITY AND PURITY) OF HEXABROMOCYCLODODECANE (HBCD) IN SUPPORT OF "A PROLONGED SEDIMENT TOXICITY TEST WITH *HYALELLA AZTECA*, AN ACTIVATED SLUDGE, RESPIRATION INHIBITION TEST AND A DETERMINATION OF THE CONTACT SENSITIZATION POTENTIAL VIA THE LOCAL LYMPH NODE ASSAY USING CBA MICE"

- I. Protocol Number: HBCD-05-29-2003
- II. Sponsor: American Chemistry Council
Brominated Flame Retardant Industry Panel
1300 Wilson Boulevard
Arlington, Virginia 22209
Study Monitor: Wendy K. Sherman
- III. Analytical Testing Facilities: Albemarle Corporation
Process Development Center
Gulf States Road
Baton Rouge, LA 70805
Study Chemist: Paul F. Ranken, Ph. D.
- IV. Date of Study Initiation: June 2, 2003
Date of Study Completion: June 27, 2003
- V. Test Article: A sample of the test article, Hexabromocyclododecane (WIL Test Substance 5850), was analyzed at the Albemarle Process Development Center. The test article is a composite of commercial product from Albemarle Corporation, Great Lakes Chemical Corporation and the Dead Sea Bromine Group. Wildlife International Ltd., Easton, MD 21601, prepared the composite.
- VI. Objective/Methodology: This study was initiated to confirm the identity of the test article and to demonstrate the composition and purity of the test article. Fourier Transform Infrared Spectroscopy using SOP No. ARS-284-R4 was used to confirm the

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identity of the sample of the test article. In this procedure, the test article infrared spectrum was compared to a standard reference spectrum of HBCD (Aldrich 1,2,5,6,9,10-Hexabromocyclododecane, tech.; The Aldrich Library of FT-IR Spectra, Volume 1, Spectrum 107A). High Performance Liquid Chromatography (HPLC) using SOP No. ARS-432-R1 and inhibitor-free tetrahydrofuran (THF) was used to determine the composition (area% of the three HBCD diastereomers) and purity (total area % HBCD; duplicate analysis) of the test article. Chain of Custody and sample handling were conducted according to established standard operating procedures.

VII. Results:

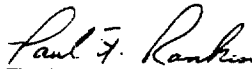
Table 1 contains the test article analytical data from the study. Fourier Transform Infrared Spectroscopy confirmed the identity of the test article. High Performance Liquid Chromatography (HPLC) showed that the test article had a purity of 95.86%. The HBCD consisted of 8 area% alpha diastereomer, 5.37 area% beta diastereomer and 86.63 area% gamma diastereomer.

VIII. Regulatory Requirements:

The study conformed to the requirements of EPA TSCA (40 CFR Part 792) Good Laboratory Practice Regulations and the OECD [C(97)186/Final] Good Laboratory Practice Regulations.

IX. Data/Record Retention:

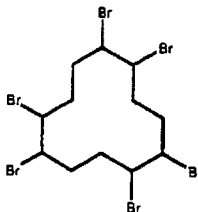
All original raw data, log books, spectra and reports will be forwarded to the Quality Assurance Unit (QAU) for a final review prior to filing in the designated Health and Environment archives at Albemarle Corporation, Health and Environment Department, 451 Florida Street, Baton Rouge, LA 70801.


Paul F. Ranken, Ph.D.
STUDY CHEMIST

June 27 2003
DATE

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TABLE 1. CONCLUSIONS AND TEST ARTICLE ANALYTICAL DATA

CHEMICAL NAME: Hexabromocyclododecane C.A.S. No.: 3194-55-6 MOLECULAR FORMULA: C ₁₂ H ₁₈ Br ₆ PHYSICAL FORM: White Powder CHEMICAL STRUCTURE:						
						
ANALYSIS		RESULTS		ANALYSIS DATES	ANALYST	
FT-IR		The sample FT-IR spectrum matched that of the Aldrich reference spectrum. All spectra are on file with the original data.			06/06/03	W. T. Cobb
HPLC		Composition (area %)	Purity (area %)	Average Purity (area %)	06/06/03	J. S. Arroyave
Alpha isomer		8.00	7.31; 8.04	7.67		
Beta isomer		5.37	5.07; 5.22	5.15		
Gamma isomer		86.63	83.81; 82.26	83.04		
		100 %		95.86 %		
CONCLUSION: Based on these analytical data, the test article was identified as HBCD having a purity of 95.86% and containing the three HBCD diastereomers.						

HEXABROMOCYCLODODECANE (HBCD):
A PROLONGED SEDIMENT TOXICITY TEST WITH
Hyalella azteca USING SPIKED SEDIMENT
WITH 2% TOTAL ORGANIC CARBON

FINAL REPORT

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 439A-119B

ASTM E 1706-95b Guideline

U. S. Environmental Protection Agency
Series 850 – Ecological Effects Test Guidelines
OPPTS Number 850.1735

AUTHORS:

Susan Thomas
Henry O. Krueger, Ph.D.
Timothy Z. Kendall

STUDY INITIATION DATE: February 5, 2002

STUDY COMPLETION DATE: August 19, 2003

SUBMITTED TO:

American Chemistry Council's
Brominated Flame Retardant Industry Panel
1300 Wilson Boulevard
Arlington, Virginia 22209

Wildlife International, Ltd.

8598 Commerce Drive
Easton, Maryland 21601
U.S.A
1-(410) 822-8600

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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

SPONSOR: American Chemistry Council's Brominated Flame Retardant Industry Panel

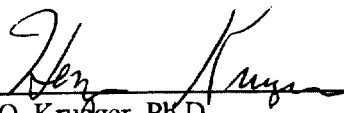
TITLE: Hexabromocyclododecane (HBCD): A Prolonged Sediment Toxicity Test with
Hyalella azteca Using Spiked Sediment with 2% Total Organic Carbon

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 439A-119B

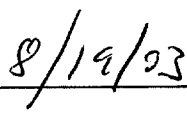
STUDY COMPLETION: August 19, 2003

This study was conducted in compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency in 40 CFR Parts 160 and 792, 17 August 1989; OECD Principles of Good Laboratory Practice (ENV/MC/CHEM (98) 17); and Japan MAFF (11 NohSan, Notification No. 6283, Agricultural Production Bureau), 1 October 1999.

STUDY DIRECTOR:



Henry O. Krueger, Ph.D.
Director of Aquatic Toxicology/ Terrestrial Plants and Insects



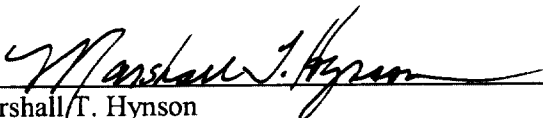
Date

- 3 -

QUALITY ASSURANCE STATEMENT

This study was examined for compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency in 40 CFR Parts 160 and 792, 17 August 1989; OECD Principles of Good Laboratory Practice (ENV/MC/CHEM (98) 17); and Japan MAFF (11 NohSan, Notification No. 6283, Agricultural Production Bureau), 1 October 1999. The dates of all inspections and audits and the dates that any findings were reported to the Study Director and Laboratory Management were as follows:

ACTIVITY:	DATE CONDUCTED:	DATE REPORTED TO:	
		STUDY DIRECTOR:	MANAGEMENT:
Initial Trial 439A-119			
Test Substance Preparation	January 10, 2003	January 13, 2003	May 29, 2003
Water Chemistry	February 10, 2003	February 10, 2003	February 12, 2003
Matrix Fortification and Observations	February 12, 2003	February 12, 2003	February 20, 2003
Second Trial 439A-119A			
Test Substance Preparation	March 21, 2003	March 21, 2003	March 25, 2003
Observations	May 6, 2003	May 6, 2003	May 7, 2003
Definitive Trial 439A-119B			
Water Chemistry	May 28, 2003	May 28, 2003	June 2, 2003
Calibration Standard Preparation	June 25, 2003	June 25, 2003	June 30, 2003
Dry Weight Measurements	June 30, 2003	June 30, 2003	July 3, 2003
Analytical Data and Draft Report	July 15, 16, 17 and 18, 2003	July 18, 2003	August 19, 2003
Biology Data and Draft Report	July 16, 17 and 18, 2003	July 18, 2003	July 25, 2003
Final Report	August 19, 2003	August 19, 2003	August 19, 2003


Marshall T. Hynson
Quality Assurance Program Supervisor

8/19/2003
Date

- 4 -

REPORT APPROVAL

SPONSOR: American Chemistry Council's Brominated Flame Retardant Industry Panel

TITLE: Hexabromocyclododecane (HBCD): A Prolonged Sediment Toxicity Test with
Hyalella azteca Using Spiked Sediment with 2% Total Organic Carbon

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 439A-119B


STUDY DIRECTOR:



Henry O. Krueger, Ph.D.
Director of Aquatic Toxicology/ Terrestrial Plants and Insects

8/19/03
Date

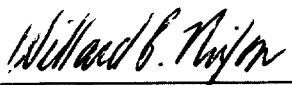
PRINCIPAL INVESTIGATOR:



Timothy Z. Kendall
Supervisor

8/19/03
Date

MANAGEMENT:



Willard B. Nixon, Ph.D.
Director of Chemistry

8/19/03
Date

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SUMMARY

SPONSOR:	American Chemistry Council's Brominated Flame Retardant Industry Panel
SPONSOR'S REPRESENTATIVE:	Wendy Sherman
LOCATION OF STUDY, RAW DATA AND A COPY OF THE FINAL REPORT:	Wildlife International, Ltd. Easton, Maryland 21601

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER:	439A-119B
TEST SUBSTANCE:	Hexabromocyclododecane (HBCD)
STUDY:	Hexabromocyclododecane (HBCD): A Prolonged Sediment Toxicity Test with <i>Hyalella azteca</i> Using Spiked Sediment with 2% Total Organic Carbon
NOMINAL SEDIMENT CONCENTRATIONS:	Negative Control, 31, 63, 125, 250, 500 and 1000 mg/Kg dry weight of sediment
TEST DATES:	Experimental Start (OECD) – January 10, 2003 Experimental Start (EPA) – May 28, 2003 Biological Termination – June 25, 2003 Analytical Termination – June 26, 2003 Experimental Termination – June 30, 2003
LENGTH OF EXPOSURE:	28 Days

TEST ORGANISM:	Amphipod (<i>Hyalella azteca</i>)
SOURCE OF TEST ORGANISMS:	Environmental Consulting and Testing 1423 N. 8 th Street Suite 118 Superior, Wisconsin 54880
AGE OF TEST ORGANISM:	13 days

28 DAY EC50:	> 1000 mg/Kg dry weight of sediment
95% CONFIDENCE LIMITS:	Not calculable
LOWEST-OBSERVED-EFFECT-CONCENTRATION:	> 1000 mg/Kg dry weight of sediment
NO-OBSERVED-EFFECT-CONCENTRATION:	1000 mg/Kg dry weight of sediment

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INTRODUCTION

This study was conducted by Wildlife International, Ltd. for American Chemistry Council's Brominated Flame Retardant Industry Panel at the Wildlife International, Ltd. aquatic toxicology facility in Easton, Maryland. An initial trial was conducted from January 15, 2003 to February 12, 2003, but was repeated due to poor survival in the control group and all treatment groups. A second trial was conducted from April 8, 2003 to May 6, 2003, but was repeated due to poor survival in the control and all treatment groups. The in-life phase of the definitive test was conducted from May 28, 2003 to June 25, 2003. Raw data generated by Wildlife International, Ltd. and a copy of the final report are filed under Project Number 439A-119B in archives located on the Wildlife International, Ltd. site.

OBJECTIVE

The objective of this study was to determine the effects of sediment-incorporated hexabromocyclododecane, also known as HBCD with a total organic carbon content of approximately 2%, on the amphipod, *Hyalella azteca*, during a 28-day exposure period under flow-through test conditions. The measured endpoints of the test were survivorship and growth as determined by dry weight measurements.

EXPERIMENTAL DESIGN

Non-GLP exploratory range-finding studies were performed with three freshwater species associated with sediment: oligochaetes (*Lumbriculus variegatus*), chironomids (*Chironomus riparius*) and amphipods (*Hyalella azteca*). All three species were tested at 50, 100, 500, 1000 mg/Kg dry sediment in two different types sediments, one with a 2% organic carbon content and the other with a 5% organic carbon content. Based on the results of the range-finding studies (Appendix 9), the amphipods were found to be the most sensitive species in both sediment types, with clear effects in the 500 mg/Kg treatment group. Therefore, more definitive GLP studies were conducted with the freshwater amphipod, *Hyalella azteca* in both sediment types. This study reports the results of HBCD exposure in the 2% organic content sediment.

Groups of amphipods were exposed to a geometric series of six test concentrations and a negative control for 28 days under flow-through test conditions. Eight replicate test compartments were maintained in each treatment and control group, with 10 amphipods in each test compartment, for a total

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of 80 amphipods per test concentration. Each test compartment contained a quantity of sediment and overlying water. Additional replicates were added in the control group, low and high treatment groups for analytical sampling of water and sediment. The “analytical” replicates sampled on Day 0 contained no amphipods, while amphipods were added at test initiation to the “analytical” replicates sampled on Day 7 and at test termination.

Nominal test concentrations selected in consultation with the sponsor were 31, 63, 125, 250, 500 and 1000 mg/Kg of sediment based on the dry weight of the sediment. The results of the study are based on the nominal test concentrations. Overlying water, pore water and sediment samples were collected and analyzed from the “analytical replicates” of the control group and the lowest and highest test concentrations. The collection and analysis were done slightly before the addition of test organisms to the test system on Day 0, on Day 7 and at the end of the test. Results of the analyses were used to confirm the lowest and highest test concentrations.

Test compartments were impartially positioned in a diluter unit approximately 2 days prior to test initiation to condition the sediment prior to introduction of organisms. Amphipods were impartially assigned to exposure compartments at test initiation. Observations of mortality and abnormal behavior were made at least three times per week during the test. Survivorship and growth (dry weights) were determined at the end of the 28-day test period. The percent reduction in the numbers of organisms present in the treatment groups at test termination in comparison to the control group was used to determine the 28-day LC50 value. The lowest-observed-effect-concentration (LOEC) and the no-observed-effect-concentration (NOEC) were determined by the concentration-response pattern and statistical analysis of the survival and dry weight data.

MATERIALS AND METHODS

The study was conducted based on the procedures outlined in the protocol, “Hexabromocyclododecane (HBCD): A Prolonged Sediment Toxicity Test with *Hyalella azteca* Using Spiked Sediment with 2% Total Organic Carbon” (Appendix 1). The protocol was based on the ASTM E 1706-95b Guideline: *Standard Test Methods for Measuring the Toxicity of Sediment-Associated Contaminants with Fresh Water Invertebrates* (1); the OPPTS 850.1735 Guideline: *Whole Sediment Acute Toxicity Invertebrates, Freshwater* (2); and *Methods for Measuring the Toxicity and Bioaccumulation of Sediment-Associated Contaminants with Freshwater Invertebrates* (2nd ed.) (3).

Test Substance

The test substance consisted of a composite of hexabromocyclododecane (CAS No. 25637-99-4) samples received from three manufacturers. The material's identity and date received from each of the manufacturers is given below:

<u>Manufacturer</u>	<u>Lot/Batch</u>	<u>Date Received</u>	Wildlife International, Ltd. <u>Identification Number</u>
Albemarle Corporation	33449-15X	December 20, 1995	3519
Great Lakes Chemical Corporation	Not Given	June 19, 1998	4515
Eurobrom b.v.	010328	December 10, 2001	5827

The composite test substance was assigned Wildlife International, Ltd. identification number 5850 and was stored under ambient conditions. The composite test substance was shipped to Albemarle Corporation for characterization and purity analyses. The results of the analyses indicated the composite test substance was homogeneous, and the test substance was assigned an expiration date of February 21, 2003. Since this test was conducted after the expiration date, another sample of the composite test substance was shipped to Albemarle Corporation to extend the expiration date. The results of the analyses indicated the composite test substance had a purity of 95.86% and a new expiration date of June 27, 2004, one year after the date of analysis (Appendix 2).

Test Sediment

Artificial sediment described as "Formulated Sediment A" in Kemble, et al. (4) was used as the test sediment. This artificial sediment is similar to that described in OECD Guideline 207 (5), but uses alpha-cellulose as its source of organic matter instead of peat moss. Alpha-cellulose was selected by Kemble, et al. (4) as a more standardized source of organic matter than peat moss.

The soil was composed of approximately 0.01% humic acid, 0.5% dolomite, 5% alpha-cellulose, 14% kaolin clay, and 80% industrial quartz sand (Appendix 3). The dry constituents of the soil were mixed in a PK-Twinshell mixer for 20 minutes. The dry soil was stored under ambient conditions until used. The soil was characterized (Agvise Laboratories, Northwood, North Dakota) as having a mean organic matter content of 3.9%, a mean organic carbon content of 2.3%, a water holding capacity of 9.3%, pH of 8.1, and particle distribution of 84% sand, 3% silt, and 13% clay.

Preparation of Test Concentrations

Concentrations of the test substance in the sediment were prepared on a dry weight basis (i.e., mg test substance/Kg dry sediment). The test substance was weighed on tared weigh paper then added directly to the sediment. The sediment and test substance were mixed for approximately 23 hours on a rotating mixer.

Eight replicate test compartments were prepared for each treatment and control group. Six additional compartments were prepared for the control, low and high treatment groups, for analytical purposes. For each replicate, approximately 100 mL of sediment was placed in a 300-mL glass beaker. Then, approximately 100 mL of overlying water was added to each replicate beaker. The beakers were then indiscriminately and slowly placed into the diluter tanks (grouped by test concentration). Two glass siphon caps were positioned in the two drains in each diluter tank to create passive flow through the holes in the sides of the beakers and exchange the water overlying the sediment. Loose plastic covers were placed over each test compartment. The sediment/water mixtures were allowed to acclimate for approximately 2 days prior to the introduction of the test organisms. At test initiation the overlying water in all test compartments appeared clear and colorless but appeared slightly cloudy and colorless at test termination.

Test Organism

The amphipod, *Hyaella azteca*, was selected as the test species for this study. Amphipods are representative of an important group of aquatic invertebrates and were selected for use in the study based upon past history of use and ease of culturing in the laboratory. The organisms were obtained from Environmental Consulting and Testing, Superior, Wisconsin. The identity of the species was verified by the supplier.

The organisms were held in water from the same source as the water used during the test. During the 6-day holding period preceding the test, water temperatures ranged from 22.3 to 23.0°C, measured with a hand-held mercury thermometer. The pH of the water ranged from 8.4 to 8.8, measured with a Fisher Scientific Accumet Model 915 pH meter. Dissolved oxygen ranged from 8.0 to 8.4 mg/L (>94% of saturation), measured with a Yellow Springs Instruments Model 51B dissolved oxygen meter.

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During holding, the amphipods appeared normal. At test initiation, amphipods from the culture were impartially added one and two at a time to each test compartment until each test compartment contained 10 organisms. All transfers were made below the water surface using wide-bore pipettes. Eight replicate test chambers in each treatment and control group contained organisms. Of the additional six analytical replicates in the control, low and high treatment groups, the two replicates sampled on Day 0 did not contain organisms, while the test chambers sampled on Day 7 and 28 did contain organisms.

Amphipods were fed daily a mixture of yeast, Cerophyll[®] and trout chow (YCT) and *Selenastrum* during the holding period, but only YCT during the test. A 1.0 mL aliquot of food was added to each test compartment daily throughout the test except on days 16, 19, 21, 23, 26 and 27. On each of these days the food was reduced due to the presence of fungal growth.

Dilution Water

The water used for culturing and testing was freshwater obtained from a well approximately 40 meters deep located on the Wildlife International, Ltd. site. The well water is characterized as moderately-hard water. The specific conductance, hardness, alkalinity and pH measurements of the well water during the four-week period immediately preceding the test are presented in Appendix IV.

The well water was passed through a sand filter to remove particles greater than approximately 25 μm , and pumped into a 37,800-L storage tank where the water was aerated with spray nozzles. Prior to use, the water again was filtered (0.45 μm) and passed through an ultraviolet sterilizer in order to remove microorganisms and fine particles. The results of periodic analyses performed to measure the concentrations of selected organic and inorganic constituents in the well water used by Wildlife International, Ltd. are presented in Appendix 5.

Test Apparatus

The test apparatus consisted of a Wildlife International, Ltd. diluter unit that held seven 52-L stainless steel diluter tanks. Siphoning systems were placed in each tank to vary the water height, allowing the dilution water to flow through the test compartments. The depth of water in one representative test compartment was 6.8 cm. The diluter was adjusted so that each test compartment received approximately two volume additions of water per day.

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Test compartments were 300-mL glass beakers with two stainless steel mesh-covered holes on opposite sides to allow for the flow of water through the test compartment. Each beaker contained approximately 100 mL of sediment and approximately 100 mL of overlying water. The depth of sediment in one representative test compartment was 2.8 cm. The depth of overlying water in each test compartment was maintained by the water levels in the diluter tanks. Test compartments were covered with plastic petri dishes and were labeled with the project number, test concentration and replicate.

Environmental Conditions

Lighting used to illuminate the culture and test chambers during holding and testing was provided by fluorescent tubes that emitted wavelengths similar to natural sunlight (Colortone® 50). A photoperiod of 16 hours of light and 8 hours of darkness was controlled with an automatic timer. A 30-minute transition period of low light intensity was provided when lights went on and off to avoid sudden changes in light intensity. Light intensity at test initiation was 123 lux at the surface of the water over one representative test chamber.

The target test temperature during the study was $23 \pm 2^{\circ}\text{C}$. Temperature was measured in the overlying water of one alternating replicate test compartment at the beginning of the test, three times per week during the test, and at the end of the test using a hand-held liquid-in-glass thermometer. Temperature also was measured continuously in the negative control diluter tank using a Fulscope ER/C Recorder. The continuous recorder was verified with a hand-held liquid-in-glass thermometer prior to test initiation and at least weekly during the test.

Hardness, alkalinity, specific conductance and ammonia measurements were made on samples of overlying water collected from one replicate test compartment of each treatment and control group at test initiation and termination. Dissolved oxygen measurements were made on samples of overlying water collected from one alternating replicate test compartment of each treatment and control group at test initiation, three times per week during the test, and at test termination. Measurements of pH were made on samples of overlying water collected from one alternating replicate test compartment of each treatment and control group at test initiation, once each week during the test, and at test termination.

Light intensity was measured using a SPER Scientific Model 840006C light meter. Dissolved oxygen was measured using a Thermo Orion model 850Aplus dissolved oxygen meter, and

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measurements of pH were made using a Thermo Orion model 525Aplus meter. Hardness and Alkalinity measurements were made by titration using procedures based on methods in *Standard Methods for the Examination of Water and Wastewater* (6). Specific Conductance was measured using a Yellow Springs Instrument Model 33 Salinity-Conductivity-Temperature meter. Ammonia was measured using a Thermo Orion Model 720Aplus pH/ISE meter.

Observations

The test chambers were observed at least three times per week to determine the number of mortalities and the number of individuals exhibiting signs of toxicity or abnormal behavior. On Day 28 of the test, amphipods were segregated from the sediment, and the numbers of live or dead amphipods were enumerated.

Dry Weight Measurements

At test termination, surviving amphipods were rinsed of excess sediment, placed in tared crucibles by replicate and dried for approximately five days at approximately 60°C. The amphipods were weighed after allowing the crucibles to cool at room temperature for at least one hour.

Statistical Analyses

The results of the test were based on the Day 0 nominal sediment concentrations. Since the percent reduction in the number of organisms present at test termination in comparison to the negative control group was less than 50% in all treatment groups, the 28-day EC50 value was estimated to be greater than 1000 mg/Kg of dry sediment, the highest concentration tested.

The no-observed-effect-concentration (NOEC) and lowest-observed-effect-concentration (LOEC) were determined by visual interpretation of the dose-response pattern and statistical analyses of the survival and dry weight data. The survival and dry weight (growth) data were evaluated for normality (Chi-Square Test) and homogeneity of variances (Bartlett's Test). After the data was deemed normal with homogeneous variance, the survival and dry weight data were analyzed using Fisher's Exact test and Bonferroni t-Test to identify those treatment levels that were statistically different ($p < 0.05$) from the negative control group (7, 8).

Collection of Analytical Samples

Analyses of sediment and water were conducted using separate test chambers collected from the control group and the lowest and highest concentration treatment groups slightly before the introduction of the organisms on Day 0, on Day 7 and at test termination on Day 28. Samples of overlying water, pore water and sediment were collected from each of the test chambers at each sampling interval. The replicates used for sample collection on Day 0 did not contain amphipods, whereas at test initiation amphipods were placed in the replicates used for sample collection on Days 7 and 28. Samples of the overlying water were collected at mid-depth in the water column, and then the remaining overlying water was poured from each test chamber. The remaining sediment was collected, centrifuged for approximately five minutes at approximately 1500 rpm, and split into separate samples of pore water and sediment. The samples were processed for analysis immediately on the day of collection, and analyzed within one day.

Analytical Methods

Concentrations of HBCD in sediment and aqueous solution samples were determined by high performance liquid chromatography using an Agilent Model 1100 High Performance Liquid Chromatograph (HPLC) equipped with an Agilent Series 1100 Variable Wavelength Detector. Chromatographic separations were achieved using a YMC-PACK ODS-AM analytical column (150 mm x 4.6 mm, 3 μ m particle size). The instrument parameters are summarized in Appendix 4.1.

The analytical methods used for the analysis of Hexabromocyclododecane (HBCD) in sediment, pore water and overlying water were developed by Wildlife International, Ltd. Samples were processed as follows:

All sediment samples were prepared by weighing 2.0 g of sediment in centrifuge tubes or scintillation vials. Quality control samples were prepared using the appropriate HBCD stock solution. Samples were sonic disrupted for approximately 10 minutes after adding 10.0 mL of tetrahydrofuran (THF). Samples were then centrifuged for approximately five minutes at approximately 1500 rpm and extract was transferred to a roundbottom flask. The extraction was repeated with an additional 10 mL of THF and centrifuged extract was added to the appropriate roundbottom flask. Each sample was rotary evaporated to approximately 0.50 – 1.0 mL using a waterbath maintained at approximately

40°C. The samples were then evaporated to dryness under a gentle stream of nitrogen. The requisite volume of THF was volumetrically added to each roundbottom flask and swirled well. If necessary, samples were filtered at this point. Secondary dilutions in 50% THF : 50% water were performed. Aliquots of each reconstituted sample were transferred to autosampler vials and submitted for HPLC/UV analysis. A method flow chart is provided in Appendix 4.2.

For aqueous solutions, QC samples were prepared by adding 50 mL of well water to a 125-mL separatory funnel and fortifying with the appropriate HBCD stock solution. Dichloromethane (DCM) (25 mL) was added to each separatory funnel. From each test solution, the requisite volume was transferred directly into a separatory funnel containing 25 mL of dichloromethane. The QC and study sample solutions were shaken (with venting) for approximately one minute and the two phases were allowed to separate. The lower organic layer was drained into a 125-mL roundbottom flask and a second 25-mL aliquot of DCM was added to the aqueous phase remaining in the funnel to perform a second extraction. The organic phase from the second extraction was combined with the organic phase from the first extraction in the same roundbottom flask. The organic extracts were rotary evaporated to 1-2 mL under vacuum and in a waterbath. The residual DCM in each flask was evaporated to dryness under a gentle stream of nitrogen. The residues were reconstituted with an appropriate volume of 50% THF : 50% water. Aliquots of the diluted extracts were transferred into an autosampler vial and submitted for HPLC/UV analysis. A method flow chart is provided in Appendix 4.13.

Calibration Curves

Five calibration standards of HBCD, ranging in concentration from 1.00 to 10.0 mg/L for both sediment and freshwater were analyzed with the sample sets. Calibration standards were prepared in 50 THF : 50% water using a stock solution of HBCD in THF (Appendix 4.3). The calibration standard series for each analysis was injected at the beginning and end of each analytical run. In addition, a minimum of one calibration standard was analyzed after every five test samples. Linear regression equations were generated using the peak area responses versus the respective concentrations of the calibration standards. The concentration of HBCD in the samples was determined by substituting the peak area responses of the samples into the applicable linear regression equation. An example of the calculations for a representative sample is included in Appendix 4.4.

Limit of Quantitation (LOQ)

The limit of quantitation (LOQ) for the overlying water samples was defined as 0.100 mg/L, calculated as the product of the concentration of the lowest calibration standard analyzed (1.00 mg/L) and the dilution factor of the matrix blank samples (0.100). The limits of quantitation (LOQ) for the pore water samples were individually calculated as the product of the concentration of the lowest calibration standard analyzed (1.00 mg/L) and the dilution factor of each test sample. The limit of quantitation (LOQ) for the sediment samples was defined as 12.5 µg/g, calculated as the product of the concentration of the lowest calibration standard analyzed (1.00 mg/L) and the dilution factor of the matrix blank samples (12.5).

Matrix Blank and Fortification Samples

One matrix blank sample for sediment was analyzed at each interval to determine possible interferences. No interferences were observed at or above the LOQ during the sample analyses (Appendix 4.5).

Sediment samples were fortified at 30.0 and 1000 µg/g using a stock solution of HBCD prepared in tetrahydrofuran (Appendix 4.3) and were analyzed concurrently with the samples. The measured concentrations for the matrix fortification samples in sediment ranged from 97.8 to 99.4% of nominal concentrations (Appendix 4.5).

One matrix blank sample for water was analyzed at each interval to determine possible interferences. No interferences were observed at or above the LOQ during the Days 0, 7 and 28 sample analyses (Appendix 4.6).

Samples of freshwater were fortified at 0.200 and 0.500 mg/L using a stock solution of HBCD prepared in tetrahydrofuran (Appendix 4.3), and were analyzed concurrently with the samples. The measured concentrations for the matrix fortification samples in freshwater ranged from 70.9 to 98.1% of nominal concentrations (Appendix 4.6).

A representative calibration curve for sediment is presented in Appendix 4.7. Representative chromatograms of low and high-level HBCD calibration standards for sediment are presented in Appendices 4.8 and 4.9, respectively. Representative chromatograms of a matrix blank sample and a

matrix fortification sample are presented in Appendices 4.10 and 4.11, respectively. A representative chromatogram of a Day 0 sediment sample is presented in Appendix 4.12.

A representative calibration curve for freshwater is presented in Appendix 4.14. Representative chromatograms of low and high-level HBCD calibration standards for freshwater are presented in Appendices 4.15 and 4.16, respectively. Representative chromatograms of a matrix blank sample and a matrix fortification sample are presented in Appendices 4.17 and 4.18, respectively. Representative chromatograms of a Day 0 overlying water sample and a Day 0 pore water sample are presented in Appendices 4.19 and 4.20, respectively.

RESULTS AND DISCUSSION

Confirmation of Test Concentrations

Results of analyses to measure concentrations of hexabromocyclododecane (HBCD) in the sediment, pore water and overlying water samples collected during the definitive test are presented in Tables 1, 2, and 3, respectively. Samples from the low and high test concentrations confirmed that the HBCD in sediment tended to remain in sediment and not move into the pore water or overlying water. The results from sediment samples from the highest test concentration (1000 mg/Kg) ranged between 82.8 and 115% of nominal, while values from the lowest concentration (31 mg/Kg) were more variable ranging from 49.5 to 125% of nominal. All samples of overlying water were below the limit of quantitation. Pore water samples from the low concentration were also below the limit of quantitation, but pore water samples from the highest test concentration had values in the low ppm range. These values are well above solubility and were believed to be the result of small particles of HBCD being extracted out of the pore water, artificially inflating the reported values.

Observations and Measurements

Measurements of temperature, dissolved oxygen and pH of the overlying water in the test chambers are presented in Tables 4, 5 and 6, respectively. Temperatures were within the $23 \pm 2^{\circ}\text{C}$ range established for the test. Dissolved oxygen concentrations were $\geq 65\%$ (5.6 mg/L) of saturation throughout the test. Gentle aeration was introduced to the test chambers on Day 0 to improve the dissolved oxygen concentration in the water overlying the sediment. Measurements of pH ranged from 7.8 to 8.6 during the test. Hardness values ranged from 130 to 140 mg/L as CaCO_3 . Alkalinity values

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ranged from 184 to 204 mg/L as CaCO₃ during the test. Conductivity ranged from 330 to 350 µmhos/cm during the test. Ammonia was measured at 0.00 mg/L as NH₃ during the test.

Observations of amphipods in individual replicates at each observation interval are presented in Appendix 7. All replicates observed during the test appeared normal, with some direct observations of mortality in the 31 and 1000 mg/Kg treatment groups and the control during the test. The presence of fungal growth was noted in all replicates, in all treatment groups and the control during the test.

The mean number of amphipods in the negative control, 31, 63, 125, 250, 500 and 1000 mg/Kg treatment groups at test termination was 7.4, 5.9, 7.8, 5.4, 6.9, 7.3 and 5.8, respectively (Table 8). The mean numbers of amphipods found in each treatment group were not found to be statistically different ($p \leq 0.05$) from the negative control group. Since the percent reduction in the number of organisms present at test termination in comparison to the negative control group was less than 50% in all treatment groups (Table 8), the 28-day EC50 value was estimated to be greater than 1000 mg/Kg of dry sediment, the highest concentration tested. The percent reduction from the control in the 31, 63, 125, 250, 500 and 1000 mg/Kg treatment groups was 20, -5.4, 27, 6.8, 1.4 and 22%, respectively. The mortality in these groups was moderate and there was no evidence of a concentration dependent response.

The average individual dry weights for the surviving amphipods in each replicate at test termination are presented in Appendix 8. The average dry weight per amphipod in the negative control group was 0.11 mg (Table 8). The average dry weight per amphipod in the 31, 63, 125, 250, 500 and 1000 mg/Kg treatment groups was 0.10, 0.13, 0.14, 0.12, 0.17 and 0.14 mg, respectively. The dry weights were not significantly different ($p > 0.05$) from the negative control weights, and any differences were not concentration-dependent. Therefore, there were no apparent effects on growth (dry weight) observed at test termination.

CONCLUSIONS

The 28-day EC50 value for amphipods (*Hyalella azteca*) exposed to hexabromocyclododecane in sediment was >1000 mg/Kg dry weight of sediment, the highest nominal concentration tested. Determination of the lowest-observed-effect-concentration (LOEC) and the no-observed-effect-concentration (NOEC) was based on an evaluation of the survival and growth (dry weight) data. Based

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on the results of this study, the LOEC was >1000 mg/Kg dry weight of sediment and the NOEC was 1000 mg/Kg dry weight of sediment.

REFERENCES

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- 6 **APHA, AWWA, WPCF.** 1998. *Standard Methods for the Examination of Water and Wastewater.* 20th Edition, American Public Health Association. American Water Works Association. Water Pollution Control Federation, New York..
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Table 1

Measured Concentrations of Hexabromocyclododecane (HBCD) in Sediment Samples from a Survival and Growth Sediment Toxicity Test with *Hyalella azteca*

Nominal Sediment Concentration (µg/g)	Sample Number (439A-119B-)	Sampling Interval (Day)	Measured HBCD Concentration (µg/g) ^{1,2}	Percent of Nominal ³
0.0 (Negative Control)	S-1	0	< LOQ	--
	S-2	0	< LOQ	--
	S-7	7	< LOQ	--
	S-8	7	< LOQ	--
	S-13	28	< LOQ	--
	S-14	28	< LOQ	--
31	S-3*	0	15.4	49.5
	S-4	0	38.7	125
	S-9	7	29.2	94.3
	S-10	7	26.4	85.0
	S-15*	28	16.5	53.2
	S-16	28	20.7	66.8
1000	S-5	0	1035	104
	S-6	0	1022	102
	S-11	7	1150	115
	S-12	7	992	99.2
	S-17	28	828	82.8
	S-18	28	1039	104

¹ The limit of quantitation (LOQ) was 12.5 µg/g calculated as the product of the concentration of the lowest calibration standard (1.00 mg/L) and the dilution factor of the matrix blank samples (12.5).

² Analytical results were generated using wet weights. The tabulated values are reported on a dry weight basis.

³ Results were generated using Excel 2000 in the full precision mode. Manual calculations may differ slightly.

* Analytical result is considered extrapolated, but quantitative.

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Table 2

Measured Concentrations of Hexabromocyclododecane (HBCD) in Overlying Water Samples from a Survival and Growth Sediment Toxicity Test with *Hyalella azteca*

Nominal Test Concentration ($\mu\text{g/L}$) ¹	Sample Number (439A-119B-)	Sampling Interval (Day)	Measured HBCD Concentration (mg/L) ^{2,3}
0.0 (Negative Control)	W-1	0	< LOQ
	W-2	0	< LOQ
	W-7	7	< LOQ
	W-8	7	< LOQ
	W-13	28	< LOQ
	W-14	28	< LOQ
31	W-3	0	< LOQ
	W-4	0	< LOQ
	W-9	7	< LOQ
	W-10	7	< LOQ
	W-15	28	< LOQ
	W-16	28	< LOQ
1000	W-5	0	< LOQ
	W-6	0	< LOQ
	W-11	7	< LOQ
	W-12	7	< LOQ
	W-17	28	< LOQ
	W-18	28	< LOQ

¹ Nominal concentration of sediment samples.

² The limit of quantitation (LOQ) was 0.100 mg/L, calculated as the product of the concentration of the lowest calibration standard (1.00 mg/L) and the dilution factor of the matrix blank samples (0.100).

³ Results were generated using Excel 2000 in the full precision mode. Manual calculations may differ slightly.

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Table 3

Measured Concentrations of Hexabromocyclododecane (HBCD) in Pore Water Samples from a Survival and Growth Sediment Toxicity Test with *Hyaella azteca*

Nominal Test Concentration ($\mu\text{g/g}$) ¹	Sample Number (439A-119B-)	Sampling Interval (Day)	Measured HBCD Concentration (mg/L) ^{2,3}
0.0 (Negative Control)	PW-1	0	< 0.476
	PW-2	0	< 0.455
	PW-7	7	< 0.476
	PW-8	7	< 0.400
	PW-13	28	< 0.417
	PW-14	28	< 0.455
31	PW-3	0	< 0.526
	PW-4	0	< 0.667
	PW-9	7	< 0.435
	PW-10	7	< 0.435
	PW-15	28	< 0.385
	PW-16	28	< 0.313
1000	PW-5	0	3.73
	PW-6*	0	4.23
	PW-11	7	2.79
	PW-12**	7	4.69
	PW-17	28	1.58
	PW-18	28	2.09

¹ Nominal concentration of sediment samples

² The limit of quantitation (LOQ) for pore water samples was calculated individually as the product of the concentration of the lowest calibration standard (1.00 mg/L) and the dilution factor of each test sample.

³ Results were generated using Excel 2000 in the full precision mode. Manual calculations may differ slightly.

* Analytical result is considered extrapolated, but quantitative

**Sample reanalyzed.

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Table 4

Temperature of Overlying Water in the Test Compartments

Nominal Concentration (mg/Kg)	Temperature (°C) ¹													
	Day		0		1		2		5		7		9	
	Replicate		A	B	A	B	C	D	D	E	E	F	F	G
Negative Control			22.8	22.8	22.8	22.8	22.9	22.9	22.9	22.8	22.8	22.8	23.0	23.3
31			22.7	22.8	22.7	22.8	22.8	22.8	22.8	22.8	22.9	22.8	23.1	22.4
63			22.7	22.7	22.7	22.8	22.8	22.8	22.8	22.9	22.9	22.8	23.1	22.5
125			22.7	22.7	22.7	22.8	22.8	22.8	22.8	22.9	22.9	22.8	23.1	22.4
250			22.8	22.8	22.8	22.8	22.9	22.9	22.9	23.0	23.0	22.9	23.3	22.8
500			22.7	22.7	22.8	22.8	22.8	22.8	22.8	22.9	22.9	22.8	23.1	22.5
1000			22.7	22.7	22.8	22.8	22.8	22.8	22.8	23.0	23.0	22.9	23.1	22.7

¹Manual temperature measurements. Temperature measured continuously during the test ranged from approximately 22.5 to 23.5, measured to the nearest 0.5°C.

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Table 5

Dissolved Oxygen in Overlying Water in the Test Compartments

Nominal Concentration (mg/Kg)	Day Replicate	Dissolved Oxygen (mg/L) ¹															
		0	1	2	5	7	9	12	14	16	19	21	23	26	27	28	
		A	B	C	D	E	F	G	H	A	B	C	D	E	F	G	
Negative Control		8.4	8.1	8.0	8.3	8.5	8.1	8.2	8.0	8.3	7.7	6.8	7.4	7.6	8.1	6.7	
31		8.4	8.3	8.1	8.1	8.6	8.1	8.0	7.2	8.2	7.7	6.5	6.9	7.5	7.3	6.8	
63		8.4	8.3	7.9	8.1	8.6	8.3	8.4	8.1	7.8	7.1	6.1	7.7	5.7	6.9	8.3	
125		8.4	8.3	8.2	7.9	8.5	8.2	8.2	8.1	8.1	8.3	8.2	7.1	8.1	6.7	7.3	
250		8.3	8.3	8.2	8.2	8.5	8.2	8.3	8.0	8.2	8.1	8.2	7.9	7.8	8.2	8.1	
500		8.4	8.1	8.1	7.9	8.5	8.2	7.8	8.1	8.1	7.8	7.7	5.6	7.9	6.4	8.3	
1000		8.5	8.2	8.2	8.1	8.5	8.2	7.7	8.0	7.1	7.8	6.9	7.2	7.6	6.6	6.1	

¹A dissolved oxygen concentration of 5.1 mg/L represents 60% saturation at 23.0°C.

¹A dissolved oxygen concentration of 5.1 mg/L represents 60% saturation at 23.0°C.

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Table 6

pH of Overlying Water in the Test Compartments

Nominal Concentration (mg/Kg)	Day: Replicate:	pH				
		0	7	14	21	28
		A	E	H	C	G
Negative Control		8.5	8.3	8.3	8.0	7.8
31		8.5	8.4	8.3	8.0	7.9
63		8.5	8.5	8.4	7.9	8.3
125		8.5	8.5	8.5	8.4	8.3
250		8.5	8.5	8.5	8.4	8.4
500		8.5	8.6	8.6	8.4	8.5
1000		8.5	8.6	8.6	8.1	7.9

Table 7

Hardness, Alkalinity, Conductivity and Ammonia of Overlying Water in Test Compartments

Nominal Concentration (mg/Kg)	Day 0 (Pooled Replicates)				Day 28 (Pooled Replicates)			
	Hardness (mg/L as CaCO ₃)	Alkalinity (mg/L as CaCO ₃)	Conductivity (µmhos/cm)	Ammonia (mg/L as NH ₃)	Hardness (mg/L as CaCO ₃)	Alkalinity (mg/L as CaCO ₃)	Conductivity (µmhos/cm)	Ammonia (mg/L as NH ₃)
Negative Control	132	186	330	0.00	132	196	330	0.00
31	132	185	330	0.00	136	201	340	0.00
63	130	186	330	0.00	132	198	330	0.00
125	132	184	330	0.00	136	198	340	0.00
250	136	186	335	0.00	136	200	345	0.00
500	130	186	335	0.00	138	204	350	0.00
1000	132	184	335	0.00	140	204	350	0.00

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Table 8

Mean Survival and Growth of Amphipods
(*Hyalella azteca*) During a 28-Day Sediment Toxicity Test

Nominal Concentration (mg/Kg)	Mean Number of Surviving Amphipods ¹	Percent Reduction in Survival ² (%)	Mean Individual Dry Weight ³ (mg)	Percent Reduction in Dry Weight ² (%)
Negative Control	7.4	--	0.11	--
31	5.9	20	0.10	4.6
63	7.8	-5.4	0.13	-22
125	5.4	27	0.14	-32
250	6.9	6.8	0.12	-8.3
500	7.3	1.4	0.17	-59
1000	5.8	22	0.14	-29

¹ Each replicate contained 10 amphipods at test initiation.

² Percent reduction was calculated in relation to the mean of the negative control.

³ There were no statistically significant differences ($p>0.05$) in the mean individual dry weight in comparison to the negative control group using the Bonferroni t-test.

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APPENDIX 1

Protocol, Protocol Amendments and Deviation

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PROTOCOL

HEXABROMOCYCLODODECANE (HBCD): A PROLONGED SEDIMENT TOXICITY
TEST WITH *Hyalella azteca* USING SPIKED SEDIMENT
WITH 2% TOTAL ORGANIC CARBON

ASTM E 1706-95b Guideline

U.S. Environmental Protection Agency
Series 850 – Ecological Effects Test Guidelines
OPPTS Number 850.1735

Submitted to

American Chemistry Council's
Brominated Flame Retardant Industry Panel
1300 Wilson Boulevard
Arlington, Virginia 22209

Wildlife International, Ltd.

8598 Commerce Drive
Easton, Maryland 21601
(410) 822-8600

January 3, 2002

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HEXABROMOCYCLODODECANE (HBCD): A PROLONGED SEDIMENT TOXICITY
TEST WITH *Hyalomma azteca* USING SPIKED SEDIMENT
WITH 2% TOTAL ORGANIC CARBON

SPONSOR: American Chemistry Council's
Brominated Flame Retardant Industry Panel
1300 Wilson Boulevard
Arlington, Virginia 22209

SPONSOR'S REPRESENTATIVE: Ms. Wendy Sherman

TESTING FACILITY: Wildlife International, Ltd.
8598 Commerce Drive
Easton, Maryland 21601

STUDY DIRECTOR: Henry O. Krueger, Ph.D.,
Director of Aquatic Toxicology & Non-Target Plants

LABORATORY MANAGEMENT: ~~Henry O. Krueger, Ph.D.~~ *W.H. Nixon, Ph.D.*
Director of Aquatic Toxicology & Non-Target Plants
Analytical Chemistry *NK 2/5/02*

FOR LABORATORY USE ONLY

Proposed Dates:	
Experimental Start Date: _____	Experimental Termination Date: _____
Project No.: <u>439A-119</u>	
Test Concentrations: _____	
Test Substance No.: <u>5850</u>	

PROTOCOL APPROVAL

<u><i>H. Krueger</i></u> STUDY DIRECTOR	<u><i>2/5/02</i></u> DATE
<u><i>W.H. Nixon</i></u> LABORATORY MANAGEMENT	<u><i>2/9/02</i></u> DATE
<u><i>Wendy K. Sherman</i></u> SPONSOR'S REPRESENTATIVE	<u><i>January 10, 2002</i></u> DATE

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INTRODUCTION

Wildlife International, Ltd. will conduct a prolonged sediment toxicity test with the amphipod, *Hyalella azteca*, for the Sponsor at the Wildlife International, Ltd. aquatic toxicology facility in Easton, Maryland. The study will be based upon the ASTM E 1706-95b Guideline: Standard Test Methods for Measuring the Toxicity of Sediment-Associated Contaminants with Fresh Water Invertebrates (1); the OPPTS 850.1735: Whole Sediment Acute Toxicity Invertebrates, Freshwater (2); and EPA Methods for Measuring the Toxicity and Bioaccumulation of Sediment-Associated Contaminants with Freshwater Invertebrates (3). Raw data for all work performed at Wildlife International, Ltd. and a copy of the final report will be filed by project number in archives located on the Wildlife International, Ltd. site, or at an alternative location to be specified in the final report.

OBJECTIVE

The objective of this study is to determine the effects of a sediment-incorporated test substance with a total organic carbon content of approximately 2%, on the amphipod, *Hyalella azteca*, during a 28 day exposure period under flow-through conditions. The measured endpoints of the test are survivorship and growth as determined by dry weight measurements.

EXPERIMENTAL DESIGN

Groups of amphipods will be exposed to a geometric series of at least five test concentrations, a negative (dilution water) control, and if necessary, a solvent control for approximately 28 days. Eight replicate test compartments will be maintained in each treatment and control group, with 10 amphipods in each compartment for a total of 80 individuals per test concentration. Each test compartment will contain a quantity of sediment and overlying water. Additional replicate test compartments may be included for analytical sampling of water and sediment. No amphipods will be placed in Day 0 replicates sampled at the beginning of the test. However, "analytical" replicates sampled at Day 7 and at the end of the test will contain amphipods. Test concentrations in the sediment will be prepared on a mg/kg dry weight basis.

Nominal test concentrations will be selected in consultation with the Sponsor, and will be based upon information such as the results of exploratory range-finding toxicity data, known toxicity data, physical/chemical properties of the test substance or other relevant information. Generally, each test substance concentration used in the definitive test will be at least 60% of the next higher

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concentration unless information concerning the concentration-effect curve indicates that a different dilution factor would be more appropriate. Overlying water, pore water, and sediment samples from the analytical sampling test compartments will be collected from the highest and lowest test concentrations. Day 0 samples may be collected slightly before organisms are added to the test system to accommodate analytical sampling schedules.

To control bias, amphipods will be impartially assigned to transfer chambers (e.g. 8 groups of 10 amphipods per chamber) which will then be assigned to exposure compartments at test initiation. No other potential sources of bias are expected to affect the results of the study. Survivorship and growth (mean individual dry weight) will be determined at the end of the test period (maximum of 28 days). LC50 values will be determined, when possible. The dose-response pattern and appropriate statistical analyses will be used to define the no-observed-effect-concentration (NOEC) and the lowest-observed-effect-concentration (LOEC). Nominal concentrations will be used to determine the LC50, NOEC and LOEC.

MATERIALS AND METHODS**Test Substance**

The test substance consisted of a composite of HBCD samples received from three manufacturers. The material's identity and date received from each of the manufacturers is given below:

<u>Manufacturer</u>	<u>Lot/Batch</u>	<u>Date Received</u>	<u>Wildlife International Ltd. Identification Number</u>
Great Lakes Chemical Corporation	Not Given	June 19, 1998	4515
Eurobrom b.v.	010328	December 10, 2001	5827
Albemarle Corporation	33449-15X	December 20, 1995	3519

The composite test substance was assigned Wildlife International Ltd. identification number 5850 and was stored under ambient conditions. A subsample of the composite test substance has been shipped to Albemarle Corporation for characterization and purity analyses. A copy of Albemarle Corporation's final report on characterization and purity determination will be included as an appendix to the results of these analyses.

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Preparation of Test Concentrations

Concentrations of the test substance in the sediment will be prepared on a dry weight basis (e.g., mg test chemical/Kg dry soil). Batch(es) of a suitable amount of dry sediment (ambient conditions) will be mixed with the test chemical for each test concentration. If necessary, the test substance will be dissolved in a volatile solvent (e.g. acetone) and mixed overnight so that the solvent can volatilize off the sediment during mixing. If an organic solvent is required, then a solvent control group will be included in the experimental design along with the negative (dilution water) control group. If used, the concentration of organic solvent will be minimized and will be the same in all treatment levels. The sediment and test substance will be mixed with a rotary mixer for an appropriate amount of time (e.g. overnight) for complete mixing to occur. Batches of treated sediment will be prepared for each treatment level and then transferred to test chambers. Approximately 100 ml of sediment will be placed in the bottom of each test compartment (300 ml beaker) and approximately 100- 150 ml of overlaying water will be slowly siphoned into the test compartment. After the test compartments are prepared, they will be transferred to a diluter unit and acclimated for approximately 48 hours before adding the test organisms. The length of the acclimation time may be shortened or lengthened based on the properties of the test substance.

Test Organism

The amphipod, *Hyalella azteca*, has been selected as the test species for this study. Amphipods represent an important group of aquatic invertebrates, and have been selected for use in the test based upon past use history and ease of culturing in the laboratory. Amphipods to be used in the test will be 7 to 14 days of age at test initiation. Amphipods will be fed 1.0 ml YCT (1.8 g/l) in a water suspension daily. The food ration will be reduced in all treatment levels and the control if fungal growth is seen in the control or any treatment group.

Specifications for acceptable levels of contaminants in YCT for amphipods have not been established. However, there are no levels of contaminants reasonably expected to be present in the diet that are considered to interfere with the purpose or conduct of the study.

Test Sediment

Artificial sediment, "Formulated Sediment A", as described in Kemble et al. (3) will be used as the test sediment. The soil will be composed of less than 1% humic acid and dolomite, 5% alpha-

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cellulose, 14% silt and clay (kaolin clay) and 80% industrial quartz sand. The dry constituents of the soil will be mixed in a PK Twinshell or equivalent mixer. Calcium carbonate will be added as needed to adjust the pH to 7.0 ± 0.5 . Total organic carbon content of the final mixture of sediment should be approximately 2%. Samples of the artificial sediment will be sent to a laboratory for analysis of total organic carbon.

Dilution Water

Water used for the holding and testing of amphipods will be obtained from a well approximately 40 meters deep located on the Wildlife International, Ltd. site. The water will be passed through a sand filter and pumped into a 37,800-L storage tank where the water will be aerated with spray nozzles. Prior to use the water will be filtered to $0.45 \mu\text{m}$ in order to remove fine particles. Water used for holding and testing is characterized as moderately hard. Typical values for hardness, alkalinity, pH and specific conductance are approximately:

Hardness, mg/L as CaCO_3	145
Alkalinity, mg/L as CaCO_3	190
pH	8.1
Specific Conductance, $\mu\text{mhos/cm}$	330

Hardness, alkalinity, pH and specific conductance will be measured weekly to monitor the consistency of the well water. Means and ranges of the measured parameters for the four-week period preceding the test will be provided in the final report. Analyses will be performed at least once annually to determine the concentrations of selected organic and inorganic constituents of the well water and results of the most recent GLP compliant analyses will be summarized in the final report.

Test Apparatus

The test apparatus will consist of a Wildlife International, Ltd. diluter unit that is designed to hold up to 14 tanks in a temperature controlled water bath. Each tank can receive renewal rates that can range from static to greater than 10 tank volume additions per day.

Test compartments will consist of 300 mL glass beakers with a nylon mesh covered hole on opposite sides of the beaker. Eight replicate beakers in each treatment group will be indiscriminately positioned in one or more diluter tanks. Beakers will be labeled with the project number, test

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concentration and replicate. Each beaker will contain approximately 100 ml of sediment and approximately 100 to 150 ml of overlying water. The water level in the beakers will be maintained by the water levels in diluter tanks. Volume additions in diluter tanks will be adjusted to result in approximately two or less volume additions per day. A glass siphon cap will be positioned in each diluter tank to create passive flow of dilution water through the holes in the sides of the beakers and exchange of the water overlying the sediment. Loose plastic covers will be placed over each test compartment during the test.

Environmental Conditions

Lighting used to illuminate the cultures and test chambers in test compartments during culturing and testing will be provided by fluorescent tubes that emit wavelengths similar to natural sunlight (e.g., Colortone® 50). A photoperiod of 16 hours of light and 8 hours of dark will be controlled with an automatic timer. A 30-minute transition period of low light intensity will be provided when lights go on and off to avoid sudden changes in light intensity. Light intensity will be measured at test initiation with a SPER Scientific Ltd. light meter or equivalent and should fall within the range of 100 to 1000 lux.

The target test temperature will be $23 \pm 2^{\circ}\text{C}$. Temperature will be measured using a mercury or alcohol thermometer in the overlying water in one replicate test chamber of each experimental group at the beginning and end of the test and at least three times per week during the test. Temperature also will be measured with a continuous recorder in a beaker of water placed adjacent to the test chambers. Recorder measurements will be verified with a hand-held thermometer prior to test initiation.

Dissolved oxygen will be measured in one replicate of each experimental group at the beginning and end of the test and at least three times per week during the test using a Yellow Springs Instrument Model 51B dissolved oxygen meter, or equivalent. In the event that dissolved oxygen levels fall below 60% saturation, dissolved oxygen measurements will be made in every test chamber. An attempt will be made to notify the Sponsor by telephone call or facsimile of the condition of the test and the measured dissolved oxygen concentrations. A judgment will be made as to the severity of the condition and its impact on the test and whether aeration is necessary. The outcome of that judgment will be documented in the study records.

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Measurements of pH, ammonia, hardness, alkalinity and specific conductance will be measured at the beginning and end of the test in a sample of the overlying water from one replicate in each experimental group. Measurements of pH also will be measured at least once per week in a sample of the overlying water from one replicate in each experimental group. A Fisher Accumet Model 915 pH meter, or equivalent will be used to measure pH. Ammonia will be measured with a ThermoOrian Model 720A Ion Selective Meter and ammonia probe, or equivalent. Hardness and alkalinity measurements will be made by titration using procedures based on methods in *Standard Methods for the Examination of Water and Wastewater* (5). Specific conductance will be measured using a Yellow-Springs Instrument Model 33 Salinity-Conductivity-Temperature meter, or equivalent.

If a treatment group reaches 100% mortality, then the dissolved oxygen, pH and temperature measurements will be collected at that time and then measurements will be discontinued for the remainder of the test.

Test Procedures and Biological Measurements

After the settling period for the water/sediment systems, one to two 7 to 14 day old amphipods will be sequentially added to a glass beaker until each beaker contains its complement of 10 individuals. The individuals then will be transferred below the air/water interface to the test compartments. Test compartments will be observed at least three times per week to make visual assessments of any abnormal behavior (e.g. leaving sediment, unusual swimming).

Observations will be made at test termination to determine the number of mortalities and the number of individuals exhibiting clinical signs of toxicity or abnormal behavior. On Day 28, amphipods will be segregated from the sediment and live and dead organisms will be enumerated. When the total number of individuals found in each replicate at test termination are fewer than the number initially placed in each replicate, then those missing will be considered dead. Dry weights will be determined for surviving amphipods in each replicate to evaluate effects on growth.

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Sampling for Analytical Measurements

If stock solutions are used to spike sediment, samples of the stock solutions will be collected and analyzed on the day the sediments are dosed. Overlying water, pore water, and sediment samples from the analytical sampling test compartments will be collected from the highest and lowest test concentrations. Day 0 samples may be collected slightly before organisms are added to the test system to accommodate analytical schedules. The overlying water will be sampled, then the remaining sediment will be collected, centrifuged, and split into separate samples of pore water and sediment. If samples are not analyzed immediately, then samples will be placed in an appropriate container and stored frozen until analyzed. Stock solution samples will be analyzed as soon as possible after collection. The sampling scheme is summarized below:

Experimental Group	PROPOSED NUMBERS OF VERIFICATION SAMPLES								
	Day 0			Day 7			Termination (28)		
	Wat	Sed	Pore	Wat	Sed	Pore	Wat	Sed	Pore
Stocks	5	-	-	-	-	-	-	-	-
Control	1	2	2	1	2	2	1	2	2
Solvent Control (if needed)	1	2	2	1	2	2	1	2	2
Level 1-Low Concentration	1	2	2	1	2	2	1	2	2
Level 2	-	-	-	-	-	-	-	-	-
Level 3	-	-	-	-	-	-	-	-	-
Level 4	-	-	-	-	-	-	-	-	-
Level 5-High Concentration	1	2	2	1	2	2	1	2	2
	9	8	8	4	8	8	4	8	8

The above numbers of samples represent those collected from the test and do not include quality control (QC) samples such as matrix blanks and fortifications prepared and analyzed during the analytical chemistry phase of the study.

Analytical Method Development and Verification

Wildlife International, Ltd. will develop appropriate analytical methods and validate them for Sponsor approval prior to their use in support of this study. If the Sponsor provides an analytical method, Wildlife International, Ltd. will demonstrate its validity to the Sponsor before being used in

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support of this study. All analytical methods accepted for use in this study will be added by protocol amendment and described in detail as an Appendix to the final report.

Analytical Chemistry

Chemical analysis of the samples will be performed by Wildlife International, Ltd. The analytical method used will be based upon chromatographic methodology provided by the Sponsor and/or developed at Wildlife International, Ltd. The methodology used to analyze the test samples will be documented in the raw data and summarized in the final report.

Data Analysis

When the dose-response pattern allows calculation of an EC50 value, the data will be analyzed using the computer software of C.E. Stephan (6). The program was designed to calculate the EC50 value and the 95% confidence interval by probit analysis, the moving average method, or binomial probability with nonlinear interpolation (7,8,9). The EC50 value will be calculated, when possible, using mortality data collected at the end of the test.

Analyses of mortality and growth (mean individual dry weight) data will be evaluated for normality and homogeneity of variances. If the data are deemed normal with homogeneous variances, hypothesis testing using analysis of variance (ANOVA) and multiple means tests (e.g., Dunnett's, Bonferroni, Scheffe) will be used. If the data fail the tests for normality or homogeneity, then data transformations will be tried in an attempt to correct the condition. When the data transformations fail to correct for non-normality or heterogeneity of variances, nonparametric procedures will be used to identify statistically significant differences among the experimental groups.

RECORDS TO BE MAINTAINED

Records to be maintained for data generated by Wildlife International, Ltd. will include, but not be limited to:

1. A copy of the signed protocol.
2. Identification and characterization of the test substance, if provided by the Sponsor.
3. Dates of initiation and termination of the test.
4. *Hyalella azteca* culture records.

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Wildlife International, Ltd.

5. Stock solution calculation and preparation, if applicable.
6. Biological observations.
7. Water chemistry results (e.g., hardness and alkalinity).
8. Statistical calculations, if applicable.
9. Test conditions (light intensity, photoperiod, etc.).
10. Calculation and preparation of test concentrations.
11. Copy of final report.

FINAL REPORT

A final report of the results of the study will be prepared by Wildlife International, Ltd. The report will include, but not be limited to, the following, when applicable:

1. Name and address of the facility performing the study.
2. Dates upon which the study was initiated and completed, and the definitive experimental start and termination dates.
3. A statement of compliance signed by the Study Director addressing any exceptions to Good Laboratory Practice Standards.
4. Objectives and procedures, as stated in the approved protocol, including all changes to the protocol.
5. The test substance identification including name, chemical abstract number or code number, strength, purity, composition, and other information provided by the Sponsor.
6. A copy of the protocol and protocol amendments.
7. Stability and solubility of the test substance under the conditions of administration, if provided by the Sponsor.
8. A description of the methods used to conduct the test.
9. A description of the test organisms, including the source, scientific name, age or life stage, feed types, light intensity and photoperiod.
10. A description of the preparation of the test solutions.
11. The methods used to allocate organisms to test chambers and begin the test, the number of organisms and compartments per chamber per treatment, and the duration of the test.
12. A description of circumstances that may have affected the quality or integrity of the data.
13. The name of the Study Director and the names of other scientists, professionals, and supervisory personnel involved in the study.

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14. A description of the transformations, calculations, and operations performed on the data, a summary and analysis of the biological data and analytical chemistry data, and a statement of the conclusions drawn from the analyses.
15. Statistical methods used to evaluate the data.
16. The signed and dated reports of each of the individual scientists or other professionals involved in the study, if applicable.
17. The location where raw data and final report are to be stored.

CHANGES TO PROTOCOL

Planned changes to the protocol will be in the form of written amendments signed by the Study Director and approved by the Sponsor's Representative. Amendments will be considered as part of the protocol and will be attached to the final protocol. Any other changes will be in the form of written deviations signed by the Study Director and filed with the raw data. All changes to the protocol will be indicated in the final report.

GOOD LABORATORY PRACTICES

This study will be conducted in accordance with Good Laboratory Practice Standards for EPA (40 CFR Part 160 and/or Part 792); OECD Principles of Good Laboratory Practice (ENV/MC/CHEM(98) 17); and Japan MAFF (59 NohSan, Notification No. 3850, Agricultural Production Bureau). Each study conducted by Wildlife International, Ltd. is routinely examined by the Wildlife International, Ltd. Quality Assurance Unit for compliance with Good Laboratory Practices, Standard Operating Procedures and the specified protocol. A statement of compliance with Good Laboratory Practices will be prepared for all portions of the study conducted by Wildlife International, Ltd. The Sponsor will be responsible for compliance with Good Laboratory Practices for procedures performed by other laboratories (e.g., residue analyses or pathology). Raw data for all work performed at Wildlife International, Ltd. and a copy of the final report will be filed by project number in archives located on the Wildlife International, Ltd. site, or at an alternative location to be specified in the final report.

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REFERENCES

- 1 **ASTM E 1706-95b.** 1995. *Standard Test Methods for Measuring the Toxicity of Sediment-Associated Contaminants with Fresh Water Invertebrates.* in 1997 Annual Book of ASTM Standards, Section 11 Water and Environmental Technology, Volume 11.05 Biological Effects and Environmental Fate; Biotechnology; Pesticides.
- 2 **U.S. Environmental Protection Agency.** 1996. Series 850 – Ecological Effects Test Guidelines (*draft*), OPPTS Number 850.1735: Whole Sediment Acute Toxicity Invertebrates, Freshwater.
- 3 **U.S. Environmental Protection Agency.** 2000. *Methods for Measuring the Toxicity and Bioaccumulation of Sediment-associated Contaminants with Freshwater Invertebrates.* (2nd ed.) Office of Research and Development Mid-Continent Ecology Division, Duluth, Minnesota and Office of Science and Technology Office of Water, Washington D.C. EPA 600-99/064.
- 4 **Kemble, N.E., F.J. Dwyer, C.G. Ingersoll, T.D. Dawson, and T.J. Norberg-King.** 1999. *Tolerance of Freshwater Test Organisms to Formulated Sediments for use as Control Materials in Whole-Sediment Toxicity Tests.* Environ. Toxicol. Chem. 18:222-230.
- 5 **APHA, AWWA, WPCF.** 1985. *Standard Methods for the Examination of Water and Wastewater.* 16th Edition, American Public Health Association. American Water Works Association. Water Pollution Control Federation, New York.
- 6 **Stephan, C.E.** 1978. U.S. EPA, Environmental Research Laboratory, Duluth, Minnesota. Personal communication.
- 7 **Thompson, W.R.** 1947. *Bacteriological Reviews.* Vol. II, No. 2. Pp. 115-145.
- 8 **Stephan, C.E.** 1977. "Methods for Calculating an LC50," *Aquatic Toxicology and Hazard Evaluations.* American Society for Testing and Materials. Publication Number STP 634, pp 65-84.
- 9 **Finney, D.J.** 1971. *Statistical Methods in Biological Assay.* Second edition. Griffin Press, London.

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Wildlife International, Ltd.

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APPENDIX I

IDENTIFICATION OF TEST SUBSTANCE BY SPONSOR

To be Completed by Sponsor

- I. Test Substance Identity (name to be used in the report): HBCD
Test Substance Sample Code or Batch Number: Wildlife International Ltd. Identification No. 5850
Test Substance Purity (% Active Ingredient): to be determined Expiration Date: _____
- II. Test Substance Characterization
Have the identity, strength, purity and composition or other characteristics which appropriately define the test substance and reference standard been determined prior to its use in this study in accordance with GLP Standards? X Yes _____ No
- III. Test Substance Storage Conditions
Please indicate the recommended storage conditions at Wildlife International, Ltd.
Ambient
Has the stability of the test substance under these storage conditions been determined in accordance with GLP Standards? _____ Yes _____ No
Other pertinent stability information: _____
- IV. Test Concentrations: _____ Adjust test concentration to 100% a.i. based upon the purity (%) given above.
_____ x _____ Do not adjust test concentration to 100% a.i. Test the material AS IS.
- V. Toxicity Information:
Mammalian: Rat LD50 _____ Mouse LD50: _____
Aquatic: Invertebrate Toxicity (EC/LC50) _____ Fish Toxicity (LC50) _____

Other Toxicity Information (including findings of chronic and subchronic tests): _____
- VI. Classification of the Compound:
_____ Insecticide _____ Herbicide _____ Fungicide
_____ Microbial Agent _____ Economic Poison
Other: Flame retardant _____

Wildlife International, Ltd.

Project Number 439A-119
Page 1 of 1

AMENDMENT TO STUDY PROTOCOL

STUDY TITLE: A Prolonged Sediment Toxicity Test with *Hyaella azteca* Using Spiked Sediment with 2% Total Organic Carbon

PROTOCOL NO.: 439/010302/HYA-SED2%/SUB439

AMENDMENT NO.: 1

SPONSOR: American Chemistry Council's
Brominated Flame Retardant Industry Panel


PROJECT NO.: 439A-119

EFFECTIVE DATE: January 10, 2003

AMENDMENT: Page 2:

ADD:	Proposed Dates:	Experimental Start Date: January 15, 2003
		Experimental Termination Date: February 12, 2003
	Test Concentrations:	31, 63, 125, 250, 500, 1000 mg of HBCD/Kg
	Test Substance No.:	5850
	Reference Substance No.:	5827

REASON: This information was not available at the time the protocol was signed by the Study Director.



STUDY DIRECTOR

10 Jan 03

DATE



LABORATORY MANAGEMENT

1/15/03

DATE

STUDY TITLE: HEXABROMOCYCLODODECANE (HBCD): A Prolonged Sediment Toxicity Test with *Hyalella azteca* Using Spiked Sediment with 2% Total Organic Carbon

AMENDMENT NO.: 2

PROJECT NO.: 439A-119

EFFECTIVE DATE: May 23, 2003

ADD: Proposed Dates: Experimental Start Date: May 28, 2003
Experimental Termination Date: June 25, 2003
Test Concentrations: 31, 63, 125, 250, 500, 1000 mg of HBCD/Kg
Test Substance No.: 5850
Reference Substance No.: 5827

REASON: The study will be repeated due to high mortality in the controls and all treatment groups.

AMENDMENT: Dilution Water, Page 6:

CHANGE: Prior to use the water will be filtered to 0.45 µm in order to remove fine particles.

To: Prior to use the water will be filtered to 0.45 µm and passed through an ultraviolet sterilizer in order to remove fine particles and microorganisms.

REASON: To add that the water will be sterilized with ultraviolet light.

AMMENDMENT: Test Apparatus, Page 6:

CHANGE: Test compartments will consist of 300 mL glass beakers with a nylon mesh covered hole on opposite sides of the beaker.

TO: Test compartments will consist of 300 mL glass beakers with a stainless steel mesh covered hole on opposite sides of the beaker.

REASON: To reflect that stainless steel screens will be used instead of nylon.

QA Reviewed by JLB 5.23.03

Wildlife International, Ltd.

Project Number 439A-119
Page 2 of 2

AMMENDMENT: Test Apparatus, Page 7:

CHANGE: Volume additions in diluter tanks will be adjusted to result in approximately two or less volume additions per day.

TO: Volume additions in diluter tanks will be adjusted to result in approximately two volume additions to each compartment per day.

REASON: To clarify that there will be two volume additions in the test compartments, not the test chambers.

AMMENDMENT: Test Apparatus, Page 7:

ADD: Gentle aeration will be applied to each test chamber during the test.

REASON: To add that each test chamber will be aerated.

AMMENDMENT: Environmental Conditions, Page 7:

CHANGE: Recorder measurements will be verified with a hand-held thermometer prior to test initiation.

TO: Recorder measurements will be verified with a hand-held thermometer prior to test initiation and weekly thereafter.

REASON: To clarify the frequency of recorder verifications.

AMMENDMENT: Environmental Conditions, Page 8:

CHANGE: Measurements of pH, ammonia, hardness, alkalinity and specific conductance will be measured at the beginning and end of the test in a sample of the overlying water from one replicate in each experimental group.

TO: Measurements of ammonia, hardness, alkalinity and specific conductance will be measured at the beginning and end of the test in a sample of the overlying water (pooled from each biological replicate) from each experimental group.

REASON: To clarify that the sample of overlying water used for analysis will come from the pooled replicates for each experimental group. A larger sample size is required for the above mentioned analysis than what can be achieved with just one replicate.

Wildlife International, Ltd.

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Page 3 of 3

AMMENDMENT: Test Procedures and Biological Measurements, Page 8:

CHANGE: After the settling period for the water sediment/systems, one to two 7 to 14 day old amphipods will be sequentially added to a glass beaker until each beaker contains its complement of 10 individuals. The individuals then will be transferred below the air/water interface to the test compartments.

TO: After the settling period for the water sediment/systems, one to two 7 to 14 day old amphipods from the culture will be sequentially added below the air/water interface to the test compartments until each test compartment contains its complement of 10 organisms.

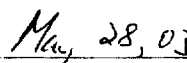
REASON: To eliminate the use of transfer beakers and in so doing reducing stress to the organisms.

AMMENDMENT: Data Analysis, Page 10:

ADD: Additional statistical analysis may be performed when appropriate. Documentation will be provided in the raw data.

REASON: To allow for additional statistical analysis when appropriate.


STUDY DIRECTOR


DATE


LABORATORY MANAGEMENT


DATE

Wildlife International, Ltd.

Project Number 439A-119
Page 1 of 1

AMENDMENT TO STUDY PROTOCOL

STUDY TITLE: HEXABROMOCYCLODODECANE (HBCD): A Prolonged Sediment
Toxicity Test with *Hyaella azteca* Using Spiked Sediment with 2% Total
Organic Carbon

PROTOCOL NO.: 439/010302/HYA-SED2%/SUB439

AMENDMENT NO.: 3

SPONSOR: American Chemistry Council's
Brominated Flame Retardant Industry Panel

PROJECT NO.: 439A-119

EFFECTIVE DATE: May 23, 2003

AMENDMENT: Page 2:

There was no reference substance used to prepare analytical standards. The standards were prepared with test substance No. 5850.

REASON: The earlier amendments were in error and should not have listed a reference substance.



STUDY DIRECTOR



DATE



LABORATORY MANAGEMENT



DATE

Reviewed by QA
JHC 7-1-03

Wildlife International, Ltd.

Project No.: 439A-119
Page 1 of 1

DEVIATION TO STUDY PROTOCOL

STUDY TITLE: A Prolonged Sediment Toxicity Test with *Hyaella azteca* Using Spiked Sediment with 2% Total Organic Carbon

PROTOCOL NO.: 439/010302/HYA-SED2%/SUB439 **DEVIATION NO.:** 1

SPONSOR: American Chemistry Council's
Brominated Flame Retardant Industry Panel **PROJECT NO.:** 439A-119B

DATE OF DEVIATION: May 26, 2003

DEVIATION: Continuous temperature measurements were made in a beaker of water adjacent to the test compartments in the negative control diluter tank instead of a beaker beside the test chambers.

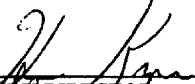
REASON: Biologist oversight. This deviation from the protocol had no adverse impact upon the results or interpretation of the study.

DEVIATION: Overlying water samples were analyzed in duplicate along with samples of pore water and sediment. The proposed schedule for sample analysis listed only one overlying water sample, instead of two.

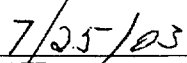
REASON: The additional samples were analyzed to show the consistency of results. This deviation from the protocol had no adverse impact on the results or interpretation of the study.

DEVIATION: Analytical method development and approval of the method by the Sponsor as stated in the protocol were not done prior to use. Additionally, the accepted methods were not amended to the protocol.

REASON: Analytical methods had been developed for soil and water on previous studies, therefore it was not necessary for the methods to be validated and approved by the Sponsor for this study. The analytical methods were not amended to the study protocol, but are fully described in the final report. This deviation from the protocol had no adverse impact on the results or interpretation of the study.



STUDY DIRECTOR



DATE



LABORATORY MANAGEMENT



DATE

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APPENDIX 2

Test Substance Characterization

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2nd 7/30/03
CERTIFIED TRUE COPY

**ALBEMARLE CORPORATION
RESEARCH AND DEVELOPMENT DEPARTMENT**

FINAL REPORT ON THE CHEMICAL CHARACTERIZATION (IDENTITY AND PURITY) OF HEXABROMOCYCLODODECANE (HBCD) IN SUPPORT OF "A PROLONGED SEDIMENT TOXICITY TEST WITH *HYALELLA AZTECA*, AN ACTIVATED SLUDGE, RESPIRATION INHIBITION TEST AND A DETERMINATION OF THE CONTACT SENSITIZATION POTENTIAL VIA THE LOCAL LYMPH NODE ASSAY USING CBA MICE"

- I. Protocol Number: HBCD-05-29-2003
- II. Sponsor: American Chemistry Council
Brominated Flame Retardant Industry Panel
1300 Wilson Boulevard
Arlington, Virginia 22209
Study Monitor: Wendy K. Sherman
- III. Analytical Testing Facilities: Albemarle Corporation
Process Development Center
Gulf States Road
Baton Rouge, LA 70805
Study Chemist: Paul F. Ranken, Ph. D.
- IV. Date of Study Initiation: June 2, 2003
Date of Study Completion: June 27, 2003
- V. Test Article: A sample of the test article, Hexabromocyclododecane (WIL Test Substance 5850), was analyzed at the Albemarle Process Development Center. The test article is a composite of commercial product from Albemarle Corporation, Great Lakes Chemical Corporation and the Dead Sea Bromine Group. Wildlife International Ltd., Easton, MD 21601, prepared the composite.
- VI. Objective/Methodology: This study was initiated to confirm the identity of the test article and to demonstrate the composition and purity of the test article. Fourier Transform Infrared Spectroscopy using SOP No. ARS-284-R4 was used to confirm the

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identity of the sample of the test article. In this procedure, the test article infrared spectrum was compared to a standard reference spectrum of HBCD (Aldrich 1,2,5,6,9,10-Hexabromocyclododecane, tech.; The Aldrich Library of FT-IR Spectra, Volume 1, Spectrum 107A). High Performance Liquid Chromatography (HPLC) using SOP No. ARS-432-R1 and inhibitor-free tetrahydrofuran (THF) was used to determine the composition (area% of the three HBCD diastereomers) and purity (total area % HBCD; duplicate analysis) of the test article. Chain of Custody and sample handling were conducted according to established standard operating procedures.

VII. Results:

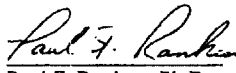
Table 1 contains the test article analytical data from the study. Fourier Transform Infrared Spectroscopy confirmed the identity of the test article. High Performance Liquid Chromatography (HPLC) showed that the test article had a purity of 95.86%. The HBCD consisted of 8 area% alpha diastereomer, 5.37 area% beta diastereomer and 86.63 area% gamma diastereomer.

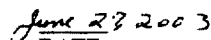
VIII. Regulatory Requirements:

The study conformed to the requirements of EPA TSCA (40 CFR Part 792) Good Laboratory Practice Regulations and the OECD [C(97)186/Final] Good Laboratory Practice Regulations.

IX. Data/Record Retention:

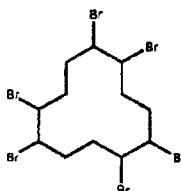
All original raw data, log books, spectra and reports will be forwarded to the Quality Assurance Unit (QAU) for a final review prior to filing in the designated Health and Environment archives at Albemarle Corporation, Health and Environment Department, 451 Florida Street, Baton Rouge, LA 70801.


Paul F. Ranken, Ph.D.
STUDY CHEMIST


DATE

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TABLE 1. CONCLUSIONS AND TEST ARTICLE ANALYTICAL DATA

CHEMICAL NAME: Hexabromocyclododecane C.A.S. No.: 3194-55-6 MOLECULAR FORMULA: C ₁₂ H ₁₈ Br ₆ PHYSICAL FORM: White Powder CHEMICAL STRUCTURE:					
					
ANALYSIS		RESULTS		ANALYSIS DATES	ANALYST
FT-IR		The sample FT-IR spectrum matched that of the Aldrich reference spectrum. All spectra are on file with the original data.			06/06/03 W. T. Cobb
HPLC		Composition (area %)	Purity (area %)	Average Purity (area %)	06/06/03 J. S. Arroyave
Alpha isomer		8.00	7.31; 8.04	7.67	
Beta isomer		5.37	5.07; 5.22	5.15	
Gamma isomer		86.63	83.81; 82.26	83.04	
		100 %		95.86 %	
CONCLUSION: Based on these analytical data, the test article was identified as HBCD having a purity of 95.86% and containing the three HBCD diastereomers.					

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APPENDIX 3

Artificial Soil Preparation

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APPENDIX 3Artificial Soil Preparation¹

Constituents	Weight (g)
Quartz Sand	32,000
Kaolin Clay	5,600
Alpha Cellulose	2,000
Dolomite	200
Humic Acid	4.0

¹ The constituents were mixed in a PK Twinshell mixer 20 minutes and the dry soil was stored under ambient conditions until used.

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APPENDIX 4

Specific Conductance, Hardness, Alkalinity and pH of Well Water Measured
During the 4-Week Period Immediately Preceding the Test

APPENDIX 4

Specific Conductance, Hardness, Alkalinity and pH of Well Water Measured
During the 4-Week Period Immediately Preceding the Test

	Mean	Range
Specific Conductance ($\mu\text{mhos/cm}$)	318 (N = 4)	310 – 320
Hardness (mg/L as CaCO_3)	130 (N = 4)	120 – 140
Alkalinity (mg/L as CaCO_3)	182 (N = 4)	182
pH	8.3 (N = 4)	8.2 – 8.4

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APPENDIX 5

Analyses of Pesticides, Organics and Metals
in Wildlife International, Ltd. Well Water

APPENDIX 5

Analyses of Pesticides, Organics and Metals in Wildlife International, Ltd. Well Water¹

Pesticides and Organics			
Component	Measured Concentration (ppb or ng/g)	Component	Measured Concentration (ppb or ng/g)
Aldicarb sulfone	< 50	Isofenphos	< 50
Aldicarb sulfoxide	< 50	Leptophos	< 50
Azinphos-ethyl	< 50	Linuron	< 50
Azinphos-methyl	< 50	Methidathion	< 50
Bifenox	< 50	Methiocarb	< 50
Bitertanol	< 50	Methomyl	< 50
Bromacil	< 50	Methoxychlor	< 250
Bromoxynil octanoic acid ester	< 50	Mirex	< 50
Captafol	< 50	Monocrotophos	< 50
Carbaryl	< 50	Myclobutanil	< 50
3-Hydroxy Carbofuran	< 50	Napropamide	< 50
Carbofuran	< 50	Norflurazon	< 50
Carbophenothion	< 50	Oxadiazon	< 50
cis-Chlordane	< 50	Oxamyl	< 50
trans-Chlordane	< 50	Oxyfluorfen	< 50
Chlorfenson	< 50	Paraoxon	< 50
trans-Chlorfenvinphos	< 50	cis-Permethrin	< 50
Chlorobenzilate	< 50	Perthane	< 50
Chloropropylate	< 50	Phosalone	< 50
Chloroxuron	< 50	Phosphamidon	< 50
Coumaphos	< 50	Piperalin	< 50
Crotoxyphos	< 50	Profenfos	< 50
Cyanazine	< 50	Promecarb	< 50
Cyfluthrin I	< 50	Propanil	< 50
Cypermethrin I	< 50	Propargite	< 50
o,p'-DDD	< 50	Propoxur	< 50
p,p'-DDE	< 50	Pyrethrin I	< 50
p,p'-DDD	< 50	Quinalphos	< 50
o,p'-DDT	< 50	Quinomethionate	< 50
p,p'-DDT	< 250	Quizalofop-ethyl	< 50
DEF	< 50	Sulprofos	< 50
Diclofop methyl	< 50	Tetrachlorovinphos	< 50
Dicrotophos	< 50	Tetradifon	< 50
Dieldrin	< 50	Thiobendazole	< 50
Diphenamid	< 50	Tilt I	< 50
Diuron	< 50	Tilt II	< 50
Endosulfan II	< 50	Trimethyl carbamate	< 50
Endrin	< 50		
Endrin ketone	< 50		
EPN	< 50		
Ethion	< 50		
Fenamiphos	< 50		
Fenarimol	< 50		
Fenobucarb	< 50		
Fenpropathrin	< 50		
Fensulfothion	< 50		
Fluzifop-P-butyl	< 50		

¹ Analyses performed by Exygen Research on samples collected on July 31, 2002.

APPENDIX 5 (continued)**Analyses of Pesticides, Organics and Metals in Wildlife International, Ltd. Well Water¹**

Metals			
	(ppm or mg/L)		(ppm or mg/L)
Aluminum	< 0.204	Manganese	< 0.0153
Arsenic	< 0.0102	Mercury	< 0.0002
Beryllium	< 0.0051	Molybdenum	< 0.0005
Cadmium	< 0.0051	Nickel	< 5.1
Calcium	28.2	Potassium	5.45
Chromium	< 0.0102	Selenium	0.009
Cobalt	< 5.1	Silver	< 0.0102
Copper	< 0.0255	Sodium	18.6
Iron	< 5.1	Zinc	< 0.0204
Magnesium	11.6		

¹Analyses performed by Exygen Research on samples collected on July 31, 2002.

APPENDIX 6

The Analysis of Hexabromocyclododecane in Water and Sediment

APPENDIX 6.1**Typical HPLC Operational Parameters for the Analysis of Hexabromocyclododecane (HBCD) in Freshwater and Sediment**

INSTRUMENT:	Agilent Model 1100 High Performance Liquid Chromatograph (HPLC) equipped with an Agilent Series 1100 Variable Wavelength Detector			
ANALYTICAL COLUMN:	YMC Pack ODS-AM Column (150 mm x 4.6 mm, 3 µm particle size)			
STOP TIME:	15 minutes			
FLOW RATE:	1.000 mL/min			
OVEN TEMPERATURE:	40°C			
SOLVENT A:	0.1% H ₃ PO ₄			
SOLVENT B:	CH ₃ CN			
GRADIENT:	Time (min.)	% A	% B	Flow (mL/min.)
	0.01	50.0	50.0	1.000
	1.00	50.0	50.0	1.000
	6.00	2.0	98.0	1.000
	11.00	2.0	98.0	1.000
	11.10	50.0	50.0	1.000
	15.00	50.0	50.0	1.000
INJECTION VOLUME:	100 µL			
APPROXIMATE HBCD RETENTION TIMES:	Sediment: 9.8 minutes Water: 10.6 minutes			
DETECTION CONDITIONS:	Wavelength - 220 nm			

Appendix 6.2

Analytical Method Flowchart for the Analysis of Hexabromocyclododecane (HBCD) in Sediment

METHOD OUTLINE FOR THE ANALYSIS OF
HEXABROMOCYCLODODECANE (HBCD) IN SEDIMENT

Rinse all glassware with THF.



Prepare samples and QC's by weighing 2.0 g of sediment into 40 mL centrifuge tubes or scintillation vials. Fortify QC's using the appropriate stock solution. Unfortified sediment will serve as the matrix blank.



Add 10.0 mL of THF and sonic disrupt each sample for approximately 10 minutes.



Centrifuge each sample for ~ 5 minutes at ~1500 rpm. Transfer extract to a roundbottom flask.



Repeat the above extraction with an additional 10 mL of THF. Add centrifuged extract to appropriate roundbottom flask.



Rotary evaporate each sample to approximately 0.50 – 1.0 mL using a waterbath maintained at approximately 40°C. Do not rotary evaporate to dryness.



Evaporate the samples to dryness under a gentle stream of nitrogen.



Volumetrically add the requisite volume of THF to each roundbottom flask and swirl well in order to dissolve all residues. Filter samples (if necessary) at this point. Perform secondary dilutions in 50% THF : 50% water.



Transfer an aliquot of each extract to an autosampler vial. Submit samples of HPLC/UV analysis.

Appendix 6.3**Analytical Stock and Standards Preparation**

Stock solutions of HBCD were prepared by weighing 0.1001 g or 0.1003 g of the test substance on an analytical balance. The test substance was transferred to a 100-mL class A volumetric flask and brought to volume using THF. The primary stock solutions contained 1.00 mg/mL of HBCD. These primary stock solutions were diluted with THF to prepare secondary stock solutions at a concentration of 0.100 mg/mL. The primary and secondary stock solutions were used to prepare matrix fortification samples. Calibration standards were prepared in 50% THF: 50% H₂O. The following shows the dilution scheme for each set of calibration standards:

Stock Concentration (mg/mL)	Aliquot (μ L)	Final Volume (mL)	Standard Concentration (mg/L)
0.100	100	10.0	1.00
0.100	250	10.0	2.50
0.100	500	10.0	5.00
0.100	750	10.0	7.50
0.100	1000	10.0	10.0

Appendix 6.4**Example Calculations for a Representative Sediment Sample**

The analytical result and percent recovery for sample number 439A-119B-S5, with a nominal concentration of 1000 µg/g, were calculated using the following equations:

$$\text{HBCD in sample (}\mu\text{g/g)} = \frac{\text{Peak Area} - \text{Y-intercept}}{\text{Slope}} \times \text{Dilution Factor} \div \text{Dry/Wet Soil Mass}$$

$$\text{Percent of nominal concentration} = \frac{\text{measured concentration of HBCD in sample (}\mu\text{g/g)}}{\text{nominal concentration of HBCD in sample (}\mu\text{g/g)}} \times 100$$

Peak Area = 45.90640

Y-intercept = -0.1449

Slope = 7.51

Dilution factor = 125

Soil = Dry Soil Mass (g)/Wet Soil Mass (g) = 9.746 g/13.16 g = 0.740

$$\text{HBCD in sample (}\mu\text{g/g)} = \frac{45.90640 + 0.1449}{7.51} \times \frac{125}{0.740}$$

$$\text{Concentration of HBCD in sample (}\mu\text{g/g)} = 1035 \mu\text{g/g}^*$$

$$\text{Percent of nominal concentration} = \frac{1035 \mu\text{g/g}}{1000 \mu\text{g/g}} \times 100$$

$$\text{Percent of nominal concentration} = 104\%^*$$

* Results were generated using Excel 2000 in the full precision mode. Manual calculations may differ slightly.

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Appendix 6.5

Matrix Blanks and Fortifications Analyzed Concurrently During Sample Analyses in Sediment

Sample Number (439A-119B-)	Sampling Interval (Day)	Concentrations of HBCD		Percent Recovery ²
		Fortified (µg/g)	Measured ¹ (µg/g)	
SMAB-1	0	0	< LOQ	--
SMAB-2	7	0	< LOQ	--
SMAB-3	28	0	< LOQ	--
SMAS-1	0	30.0	29.4	97.9
SMAS-2	0	1000	989	98.9
SMAS-3	7	30.0	29.3	97.8
SMAS-4	7	1000	994	99.4
SMAS-5	28	30.0	29.5	98.2
SMAS-6	28	1000	988	98.8
Mean =				98.5
Standard Deviation =				0.63
CV =				0.64%

¹ The limit of quantitation (LOQ) was 12.5 µg/g, calculated as the product of the concentration of the lowest calibration standard (1.00 mg/L) and the dilution factor of the matrix blank samples (12.5).

² Results were generated using Excel 2000 in the full precision mode. Manual calculations may differ slightly.

Appendix 6.6

Matrix Blanks and Fortifications Analyzed Concurrently During Sample Analyses in Freshwater

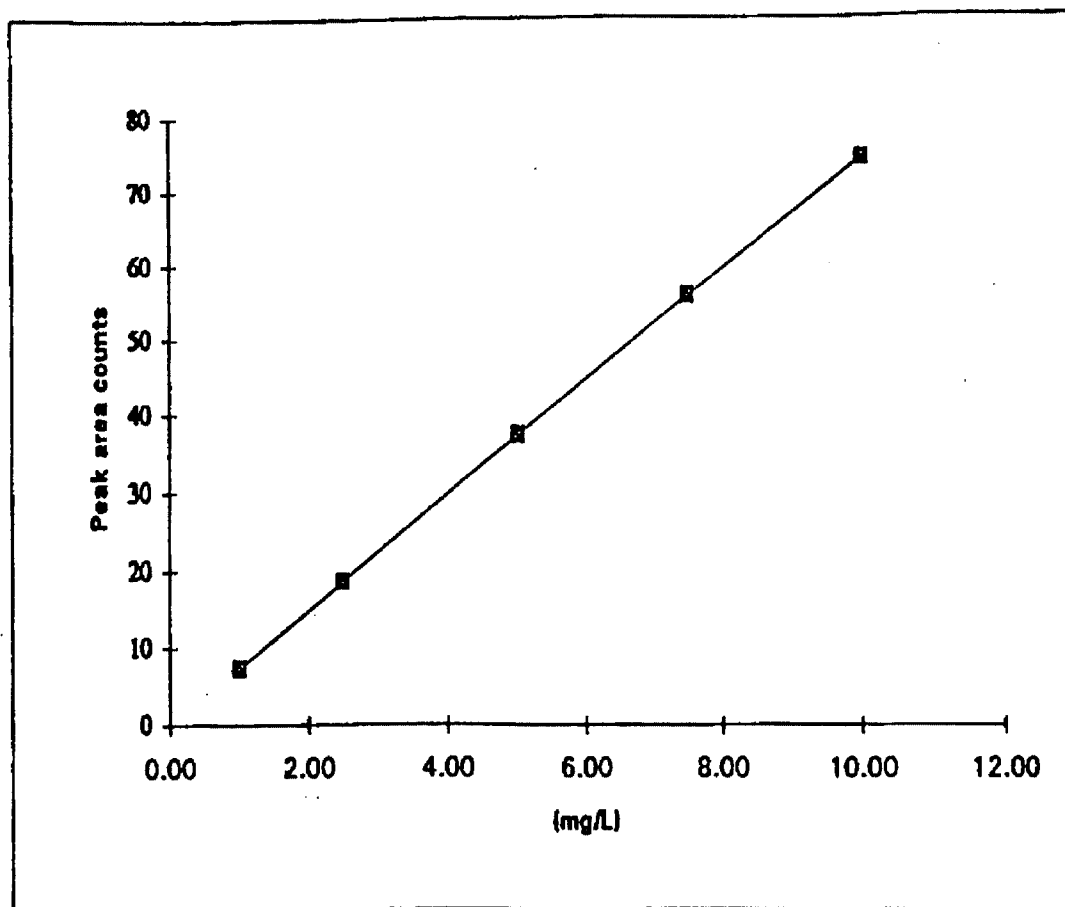
Sample Number (439A-119B-)	Sampling Interval (Day)	Concentrations of HBCD		Percent Recovery ²
		Fortified (mg/L)	Measured ¹ (mg/L)	
WMAB-1	0	0	< LOQ	--
WMAB-2	7	0	< LOQ	--
WMAB-3	28	0	< LOQ	--
WMAS-1	0	0.200	0.196	98.1
WMAS-2	0	0.500	0.436	87.2
WMAS-3	7	0.200	0.193	96.5
WMAS-4	7	0.500	0.437	87.5
WMAS-5	28	0.200	0.182	91.1
WMAS-6	28	0.500	0.354	70.9
Mean =				88.6
Standard Deviation =				9.75
CV =				11.0%

¹ The limit of quantitation (LOQ) was 0.100 mg/L, calculated as the product of the concentration of the lowest calibration standard (1.00 mg/L) and the dilution factor of the matrix blank samples (0.100).

² Results were generated using Excel 2000 in the full precision mode. Manual calculations may differ slightly.

Appendix 6.7

Representative Calibration Curve for Hexabromocyclododecane (HBCD) for Sediment Analysis

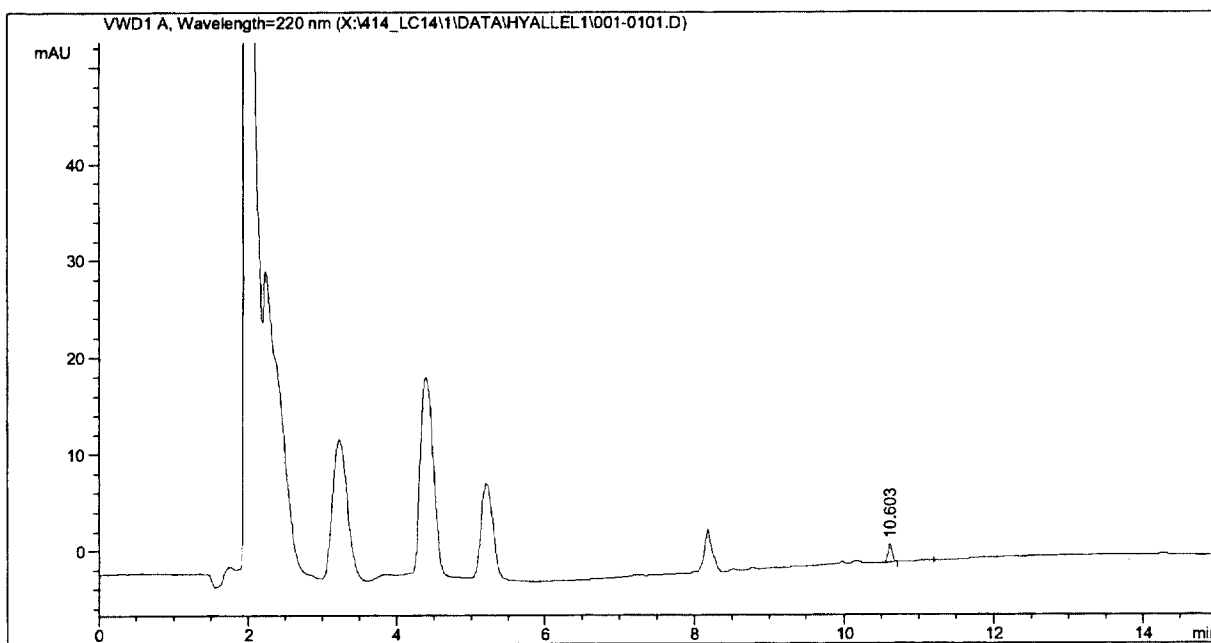


Slope = 7.51; Y-Intercept = -0.1449; $R^2 = 1.0000$

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Appendix 6.8

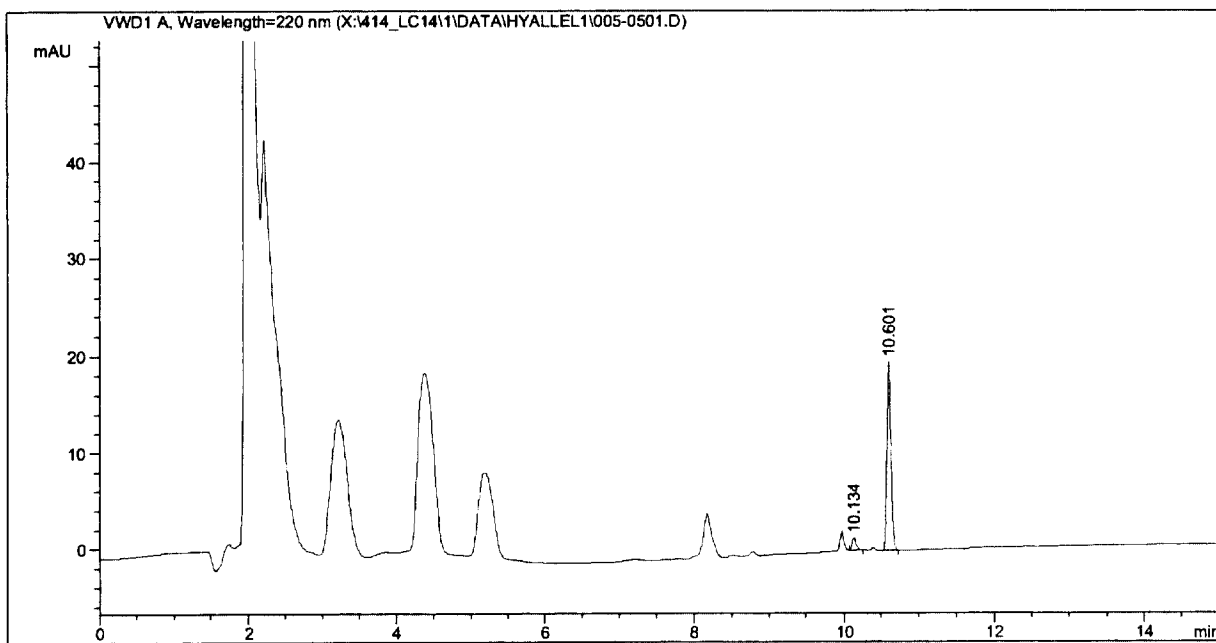
Chromatogram of a Low-level Hexabromocyclododecane (HBCD) Calibration Standard for Sediment Analysis



Nominal concentration: 1.00 mg/L

Appendix 6.9

Chromatogram of a High-level Hexabromocyclododecane (HBCD) Calibration Standard for Sediment Analysis

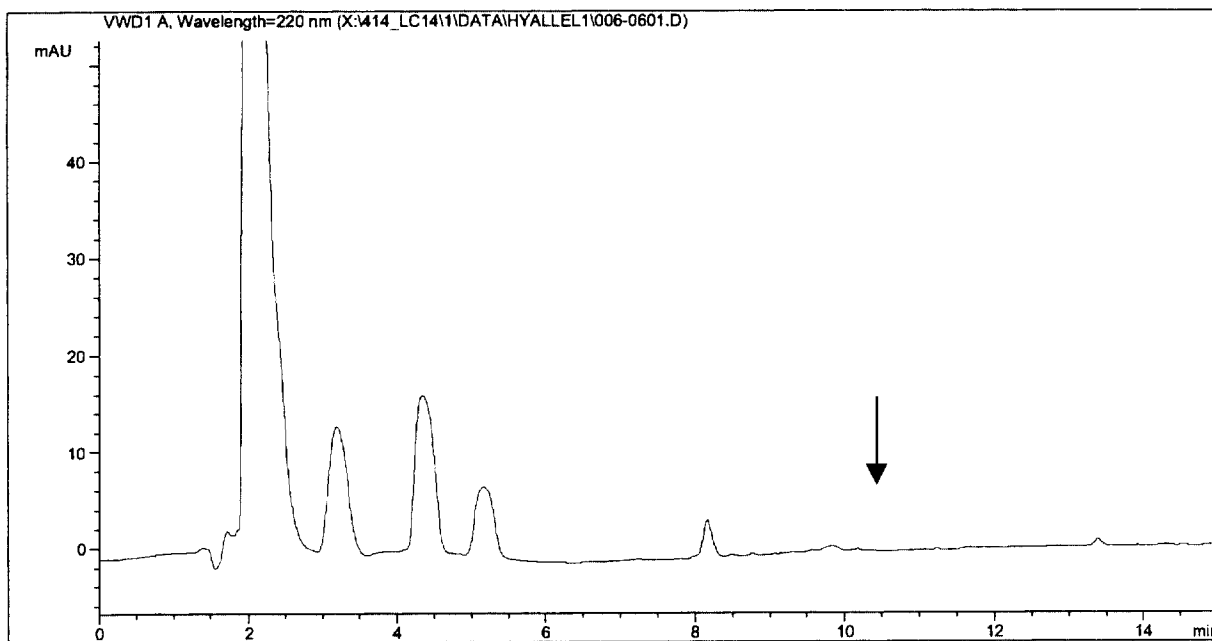


Nominal concentration: 10.0 mg/L

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Appendix 6.10

Chromatogram of a Matrix Blank for Sediment

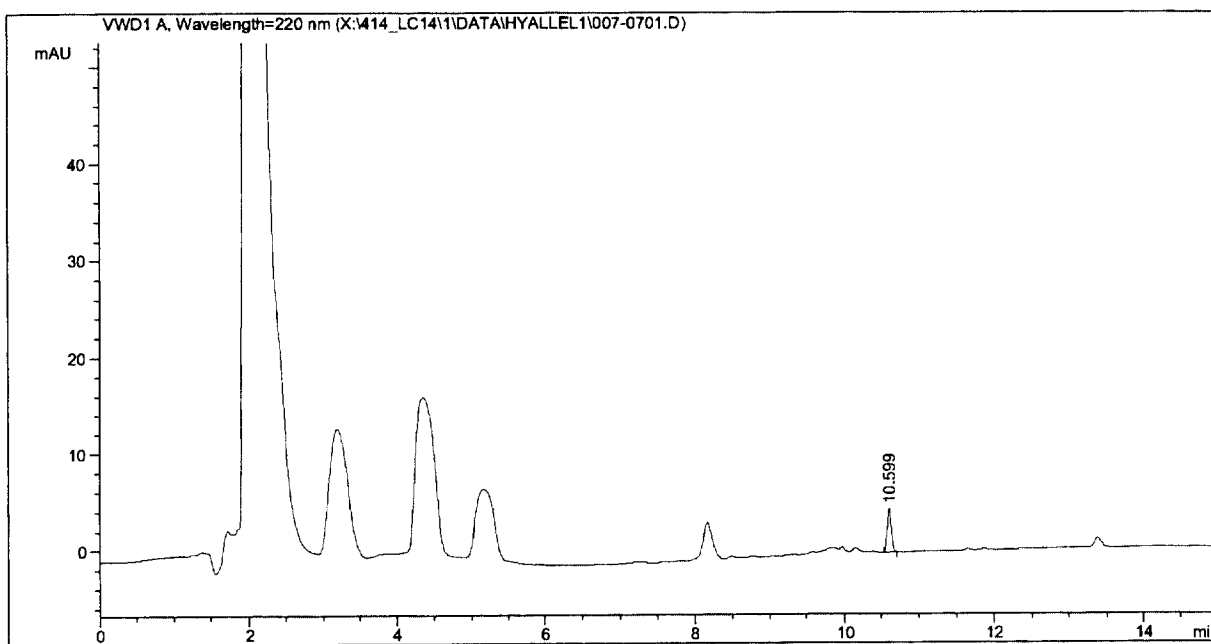


Sample number 439A-119B-SMAB-1. Arrow indicates the approximate retention time of Hexabromocyclododecane (HBCD) for sediment analysis.

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Appendix 6.11

Chromatogram of a Matrix Fortification in Sediment

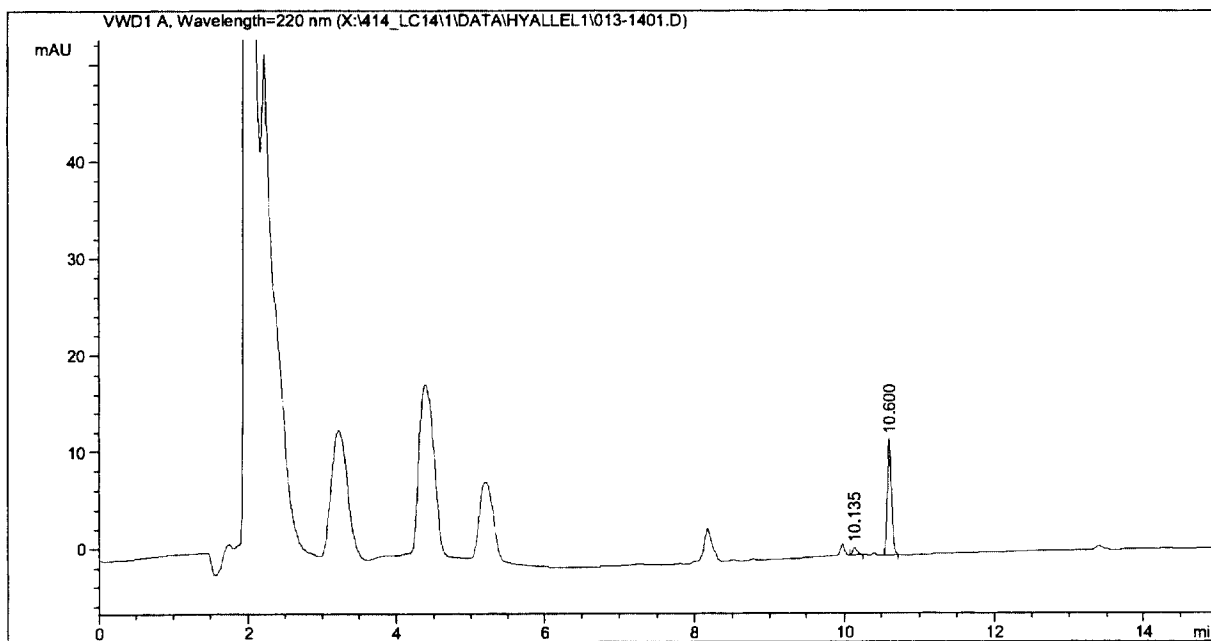


Sample number 439A-119B-SMAS-1, 30.0 $\mu\text{g/g}$, nominal concentration.

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Appendix 6.12

Chromatogram of a Day 0 Sediment Sample



Sample number 439A-119B-S5; 1000 µg/g nominal concentration.

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Appendix 6.13

**Analytical Method Flowchart for the Analysis of Hexabromocyclododecane (HBCD)
in Aqueous Solutions**

**METHOD OUTLINE FOR THE ANALYSIS OF
HEXABROMOCYCLODODECANE (HBCD) IN AQUEOUS SOLUTIONS**

Prepare each quality control (QC) sample by adding 50 mL of well water to a 125-mL separatory funnel. Fortify each QC sample with the appropriate HBCD stock solution. The matrix blank sample will not be fortified. Add 25 mL of dichloromethane (DCM) to each separatory funnel



Transfer the requisite volume of each solution directly into a separatory funnel containing 25 mL of dichloromethane (DCM).



Shake the QC and study sample solutions (with venting) for approximately one minute and allow the two phases to separate.



For each sample, drain the organic (lower) phase into a 125-mL roundbottom flask. Add a second 25-mL aliquot of DCM to the aqueous phase remaining in the funnel and perform a second extraction.



For each sample, combine the organic phase from the second extraction with the organic phase of the first extraction in the same roundbottom flask.



Rotary evaporate the organic extracts to 1-2 mL under vacuum in a waterbath set at approximately 40°C.



Evaporate the residual DCM in each flask to dryness under a gentle stream of nitrogen. Reconstitute the residues with an appropriate volume of 50% THF : 50% water.

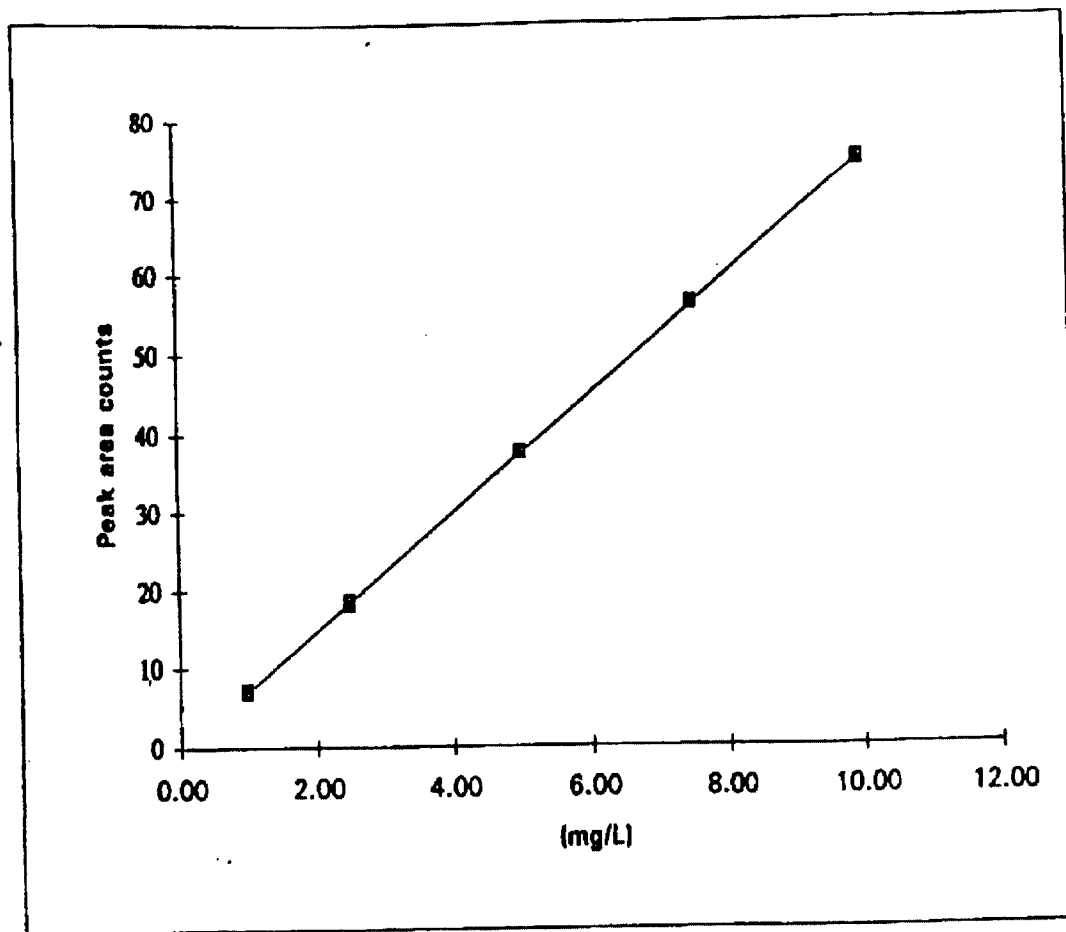


Transfer an aliquot of the diluted extracts into an autosampler vial. Submit for HPLC/UV analysis.

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Appendix 6.14

Representative Calibration Curve for Hexabromocyclododecane (HBCD) for Freshwater Analysis

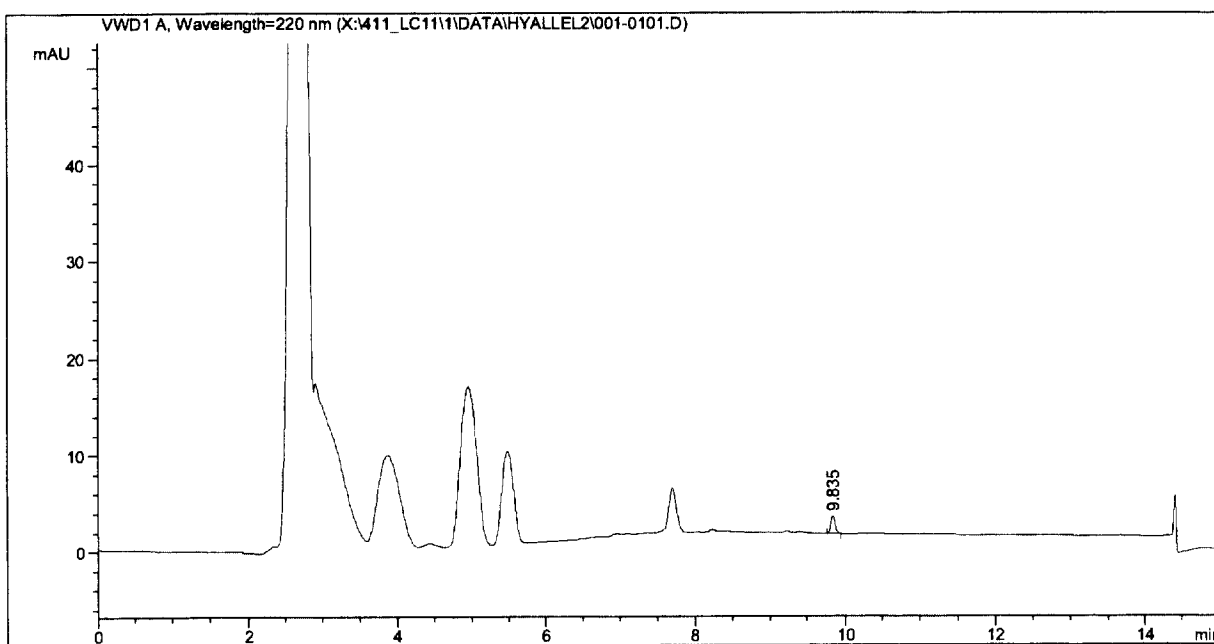


Slope = 7.53 Y-Intercept = -0.3784; $R^2 = 1.0000$

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Appendix 6.15

Chromatogram of a Low-level Hexabromocyclododecane (HBCD) Calibration Standard for
Freshwater Analysis

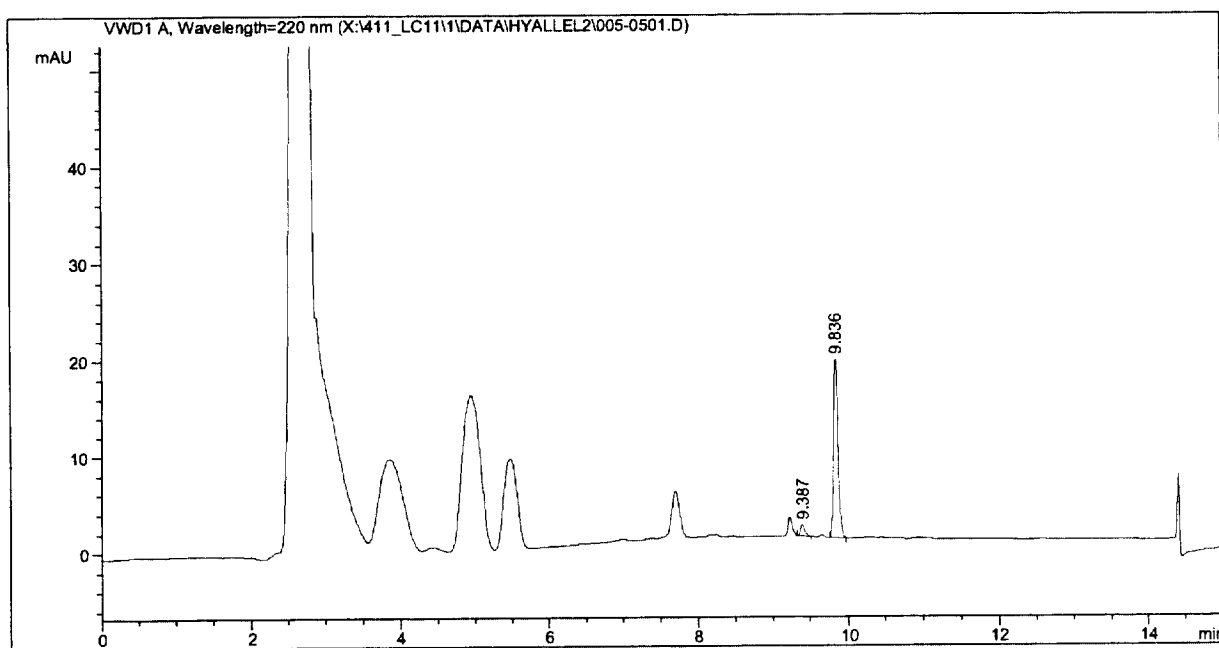


Nominal concentration: 1.00 mg/L

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Appendix 6.16

Chromatogram of a High-level Hexabromocyclododecane (HBCD) Calibration Standard for Freshwater Analysis

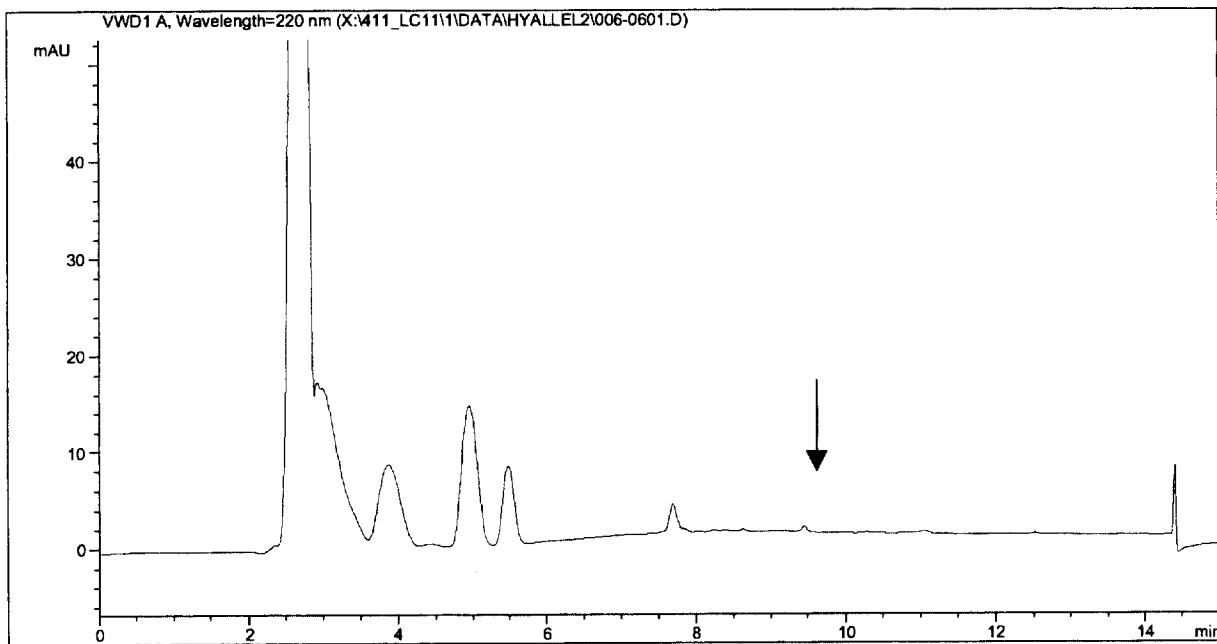


Nominal concentration: 10.0 mg/L

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Appendix 6.17

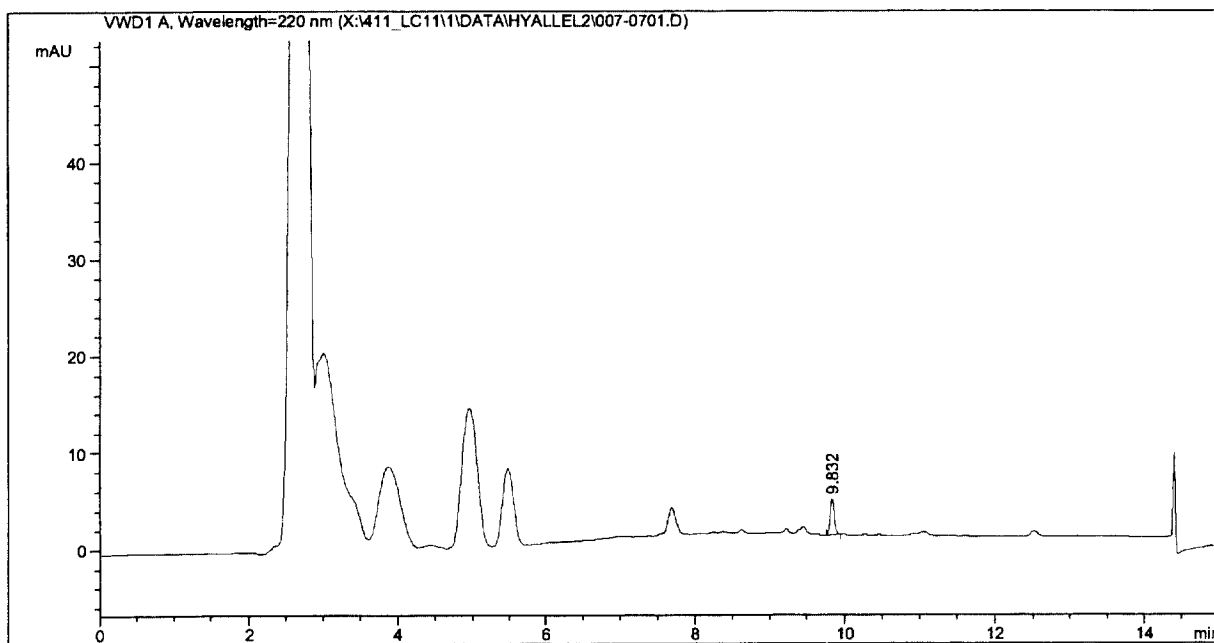
Chromatogram of a Matrix Blank for Freshwater



Sample number 439A-119B-MAB-1. Arrow indicates the approximate retention time of Hexabromocyclododecane (HBCD) for freshwater analyses.

Appendix 6.18

Chromatogram of a Matrix Fortification in Freshwater

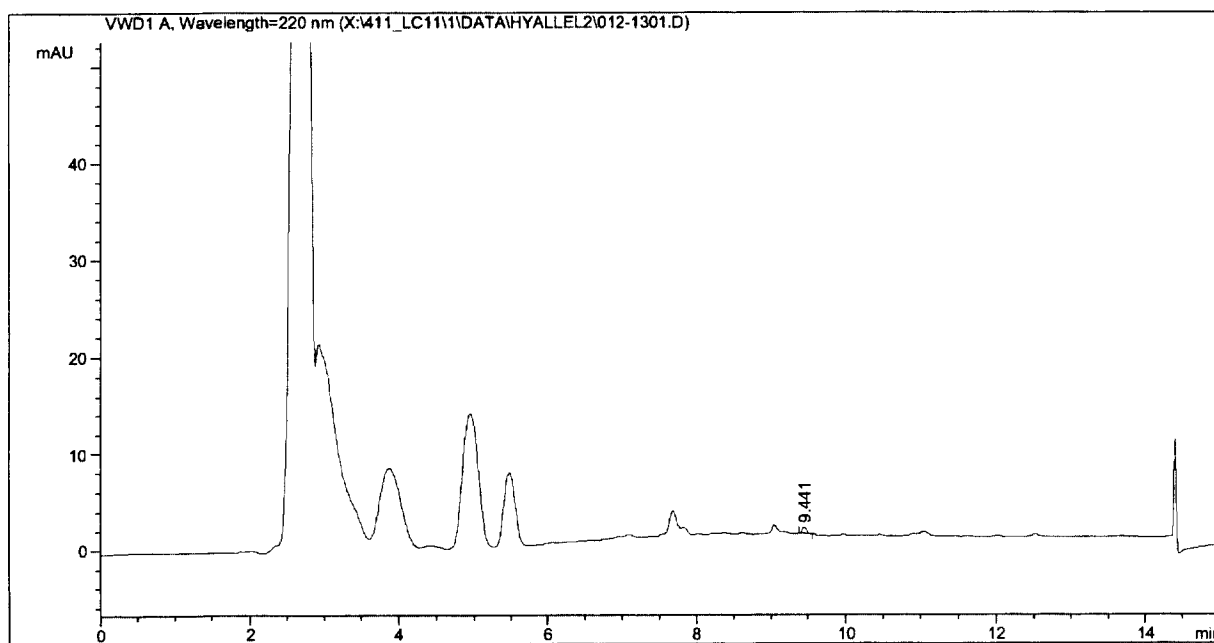


Sample number 439A-119B-MAS-1; 0.200 mg/L nominal concentration.

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Appendix 6.19

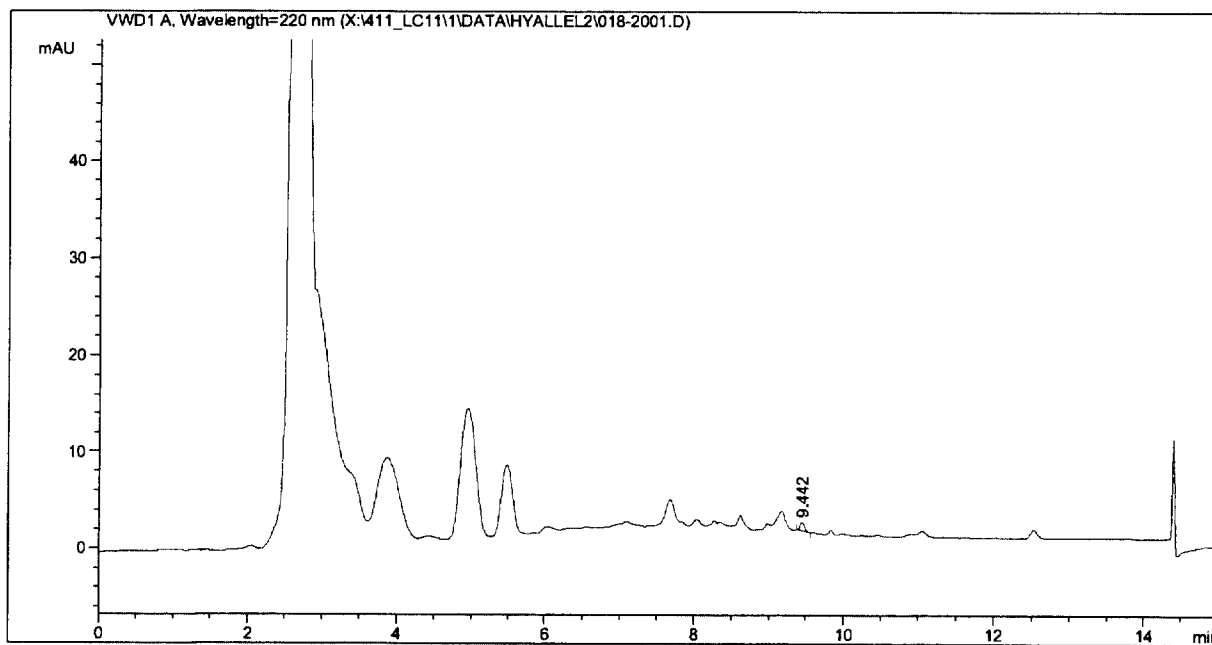
Chromatogram of a Day 0 Overlying Water Sample



Sample number 439A-119B-W4; 31 $\mu\text{g/g}$ nominal concentration.

Appendix 6.20

Chromatogram of a Day 0 Pore Water Sample



Sample number 439A-119B-PW4; 31 µg/g nominal concentration.

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APPENDIX 7

Observations of Mortality and Effects by Replicate in a 28-Day
Sediment Toxicity Test with the Amphipod *Hyaella azteca*

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APPENDIX 7

Page 1

Observations of Mortality and Effects by Replicate in a 28-Day Sediment Toxicity Test with the Amphipod *Hyalella azteca*

Nominal Concentration (mg/Kg)	Replicate	Number Exposed	Day 0		Day 1		Day 2		Day 5		Day 7		Day 9	
			No. Dead	Obs. ¹	No. Dead	Obs.	No. Dead	Obs.	No. Dead	Obs.	No. Dead	Obs.	No. Dead	Obs.
Negative Control	A	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	B	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	C	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	D	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	E	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	F	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	G	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	H	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
31	A	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	B	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	C	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	D	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	E	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	F	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	G	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	H	10	0	AN	0	AN	0	AN	0	AN	1	AN	0	AN

¹ Observations: AN = appear normal.

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APPENDIX 7 (continued)

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Observations of Mortality and Effects by Replicate in a 28-Day Sediment Toxicity Test with the Amphipod *Hyalella azteca*

Nominal Concentration (mg/Kg)	Replicate	Number Exposed	Day 0		Day 1		Day 2		Day 5		Day 7		Day 9	
			No. Dead	Obs. ¹	No. Dead	Obs.	No. Dead	Obs.	No. Dead	Obs.	No. Dead	Obs.	No. Dead	Obs.
63	A	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	B	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	C	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	D	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	E	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	F	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	G	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	H	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
125	A	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	B	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	C	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	D	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	E	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	F	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	G	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	H	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN

¹ Observations: AN = appear normal.

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APPENDIX 7 (continued)

Page 3

Observations of Mortality and Effects by Replicate in a 28-Day Sediment Toxicity Test with the Amphipod *Hyalella azteca*

Nominal Concentration (mg/Kg)	Replicate	Number Exposed	Day 0		Day 1		Day 2		Day 5		Day 7		Day 9	
			No. Dead	Obs. ¹	No. Dead	Obs.	No. Dead	Obs.	No. Dead	Obs.	No. Dead	Obs.	No. Dead	Obs.
250	A	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	B	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	C	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	D	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	E	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	F	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	G	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	H	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
500	A	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	B	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	C	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	D	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	E	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	F	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	G	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	H	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN

¹ Observations: AN = appear normal.

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APPENDIX 7 (continued)

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Observations of Mortality and Effects by Replicate in a 28-Day Sediment Toxicity Test with the Amphipod *Hyalella azteca*

Nominal Concentration (mg/Kg)	Replicate	Number Exposed	Day 0		Day 1		Day 2		Day 5		Day 7		Day 9	
			No. Dead	Obs. ¹	No. Dead	Obs.	No. Dead	Obs.	No. Dead	Obs.	No. Dead	Obs.	No. Dead	Obs.
1000	A	10	0	AN	0	AN	0	AN	0	AN	1	AN	0	AN
	B	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	C	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	D	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	E	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	F	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	G	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	H	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN

¹ Observations: AN = appear normal.

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APPENDIX 7 (continued)

Page 5

Observations of Mortality and Effects by Replicate in a 28-Day Sediment Toxicity Test with the Amphipod (*Hyalella azteca*)

Nominal Concentration (mg/Kg)	Replicate	Day 12		Day 14		Day 16		Day 19		Day 21		Day 23	
		No.	Dead	Obs. ¹	No.	Dead	Obs.	No.	Dead	Obs.	No.	Dead	Obs.
Negative Control	A	0	0	AN	0	0	AN	0	0	AN ²	0	0	AN ²
	B	0	0	AN	0	0	AN	0	0	AN ²	0	0	AN ²
	C	0	0	AN	0	0	AN	0	0	AN ²	0	0	AN ²
	D	0	0	AN	0	0	AN	0	0	AN ²	0	0	AN ²
	E	0	0	AN	0	0	AN	0	0	AN ²	0	0	AN ²
	F	0	0	AN	0	0	AN	0	0	AN ²	0	0	AN ²
	G	0	0	AN	0	0	AN	0	0	AN ²	0	0	AN ²
	H	0	0	AN	0	0	AN	0	0	AN ²	0	0	AN ²
31	A	0	0	AN	0	0	AN	0	0	AN ²	0	0	AN ²
	B	0	0	AN	0	0	AN	0	0	AN ²	0	0	AN ²
	C	0	0	AN	0	0	AN	0	0	AN ²	0	0	AN ²
	D	0	0	AN	0	0	AN	0	0	AN ²	0	0	AN ²
	E	0	0	AN	0	0	AN	0	0	AN ²	0	0	AN ²
	F	0	0	AN	0	0	AN	0	0	AN ²	0	0	AN ²
	G	0	0	AN	0	0	AN	0	0	AN ²	0	0	AN ²
	H	0	0	AN	0	0	AN	0	0	AN ²	0	0	AN ²

¹ Observations: AN = appear normal.² Fungal growth present in this test compartment.

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APPENDIX 7 (continued)

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Observations of Mortality and Effects by Replicate in a 28-Day Sediment Toxicity Test with the Amphipod *Hyalella azteca*

Nominal Concentration (mg/Kg)	Replicate	Day 12		Day 14		Day 16		Day 19		Day 21		Day 23	
		No.	Obs. ¹	No.	Obs.	No.	Obs.	No.	Obs.	No.	Obs.	No.	Obs.
63	A	0	AN	0	AN	0	AN	0	AN ²	0	AN ²	0	AN ²
	B	0	AN	0	AN	0	AN	0	AN ²	0	AN ²	0	AN ²
	C	0	AN	0	AN	0	AN	0	AN ²	0	AN ²	0	AN ²
	D	0	AN	0	AN	0	AN	0	AN ²	0	AN ²	0	AN
	E	0	AN	0	AN	0	AN	0	AN	0	AN ²	0	AN ²
	F	0	AN	0	AN	0	AN	0	AN	0	AN ²	0	AN
	G	0	AN	0	AN	0	AN	0	AN	0	AN ²	0	AN
	H	0	AN	0	AN	0	AN	0	AN ²	0	AN ²	0	AN
125	A	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	B	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	C	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	D	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	E	0	AN	0	AN	0	AN	0	AN ²	0	AN ²	0	AN
	F	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	G	0	AN	0	AN	0	AN	0	AN ²	0	AN ²	0	AN ²
	H	0	AN	0	AN	0	AN	0	AN ²	0	AN ²	0	AN

¹ Observations: AN = appear normal.² Fungal growth present in this test compartment.

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APPENDIX 7 (continued)

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Observations of Mortality and Effects by Replicate in a 28-Day Sediment Toxicity Test with the Amphipod *Hyalella azteca*

Nominal Concentration (mg/Kg)	Replicate	Day 12		Day 14		Day 16		Day 19		Day 21		Day 23	
		No. Dead	Obs. ¹	No. Dead	Obs.	No. Dead	Obs.	No. Dead	Obs.	No. Dead	Obs.	No. Dead	Obs.
250	A	0	AN	0	AN	0	AN	0	AN ²	0	AN ²	0	AN
	B	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	C	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	D	0	AN	0	AN	0	AN	0	AN ²	0	AN ²	0	AN
	E	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	F	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	G	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	H	0	AN	0	AN	0	AN	0	AN ²	0	AN ²	0	AN
500	A	0	AN	0	AN	0	AN	0	AN ²	0	AN ²	0	AN ²
	B	0	AN	0	AN	0	AN	0	AN ²	0	AN ²	0	AN ²
	C	0	AN	0	AN	0	AN	0	AN ²	0	AN ²	0	AN ²
	D	0	AN	0	AN	0	AN	0	AN ²	0	AN ²	0	AN ²
	E	0	AN	0	AN	0	AN	0	AN ²	0	AN ²	0	AN ²
	F	0	AN	0	AN	0	AN	0	AN ²	0	AN ²	0	AN ²
	G	0	AN	0	AN	0	AN	0	AN ²	0	AN ²	0	AN ²
	H	0	AN	0	AN	0	AN	0	AN ²	0	AN ²	0	AN ²

¹ Observations: AN = appear normal.² Fungal growth present in this test compartment.

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APPENDIX 7 (continued)

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Observations of Mortality and Effects by Replicate in a 28-Day Sediment Toxicity Test with the Amphipod *Hyalella azteca*

Nominal Concentration (mg/Kg)	Replicate	Day 12		Day 14		Day 16		Day 19		Day 21		Day 23	
		No. Dead	Obs. ¹	No. Dead	Obs.	No. Dead	Obs.	No. Dead	Obs.	No. Dead	Obs.	No. Dead	Obs.
1000	A	0	AN	0	AN	0	AN ²	0	AN ²	0	AN ²	0	AN ²
	B	0	AN	0	AN	0	AN ²	0	AN ²	0	AN ²	0	AN ²
	C	0	AN	0	AN	0	AN ²	0	AN ²	0	AN ²	0	AN ²
	D	0	AN	0	AN	0	AN ²	0	AN ²	0	AN ²	0	AN ²
	E	0	AN	0	AN	0	AN ²	0	AN ²	0	AN ²	0	AN ²
	F	0	AN	0	AN	0	AN ²	0	AN ²	0	AN ²	0	AN ²
	G	0	AN	0	AN	0	AN ²	0	AN ²	0	AN ²	0	AN ²
	H	0	AN	0	AN	0	AN ²	0	AN ²	0	AN ²	0	AN ²

¹ Observations: AN= appear normal.² Fungal growth present in this test compartment.

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APPENDIX 7 (continued)

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Observations of Mortality and Effects by Replicate in a 28-Day Sediment Toxicity Test with the Amphipod *Hyalella azteca*

Nominal Concentration (mg/Kg)	Replicate	Day 26		Day 27		No. Dead or MAD ³	Day 28	
		No. Dead	Obs. ¹	No. Dead	Obs.		Obs.	No. Survivors
Negative Control	A	0	AN ²	0	AN ²	4	AN	6
	B	0	AN ²	0	AN	3	AN	7
	C	0	AN ²	0	AN	3	AN	7
	D	0	AN ²	0	AN ²	4	AN	6
	E	0	AN ²	0	AN	3	AN	7
	F	0	AN ²	0	AN	1	AN	9
	G	0	AN ²	0	AN	2	AN	8
	H	0	AN ²	0	AN ²	1	AN	9
							Mean:	7.4
31	A	0	AN ²	0	AN ²	4	AN	6
	B	0	AN ²	0	AN ²	7	AN	3
	C	0	AN ²	0	AN ²	5	AN	5
	D	0	AN ²	0	AN ²	2	AN	8
	E	0	AN ²	0	AN ²	4	AN	6
	F	0	AN ²	0	AN ²	3	AN	7
	G	0	AN ²	0	AN ²	3	AN	7
	H	0	AN ²	0	AN ²	4	AN	5
							Mean:	5.9

¹ Observations: AN = appear normal.² Fungal growth present in this test compartment.³ MAD = missing and assumed dead.

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APPENDIX 7 (continued)

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Observations of Mortality and Effects by Replicate in a 28-Day Sediment Toxicity Test with the Amphipod *Hyaletta azteca*

Nominal Concentration (mg/Kg)	Replicate	Day 26		Day 27		No. Dead or MAD ³	Day 28	
		No. Dead	Obs. ¹	No. Dead	Obs.		Obs.	No. Survivors
63	A	0	AN ²	0	AN ²	3	AN	7
	B	0	AN ²	0	AN ²	4	AN	6
	C	0	AN ²	0	AN ²	3	AN	7
	D	0	AN ²	0	AN ²	0	AN	10
	E	0	AN ²	0	AN ²	3	AN	7
	F	0	AN ²	0	AN ²	1	AN	9
	G	0	AN ²	0	AN ²	3	AN	7
	H	0	AN ²	0	AN ²	1	AN	9
						Mean:		7.8
125	A	0	AN ²	0	AN ²	5	AN	5
	B	0	AN ²	0	AN ²	3	AN	7
	C	0	AN ²	0	AN ²	4	AN	6
	D	0	AN ²	0	AN ²	7	AN	3
	E	0	AN ²	0	AN ²	4	AN	6
	F	0	AN ²	0	AN ²	3	AN	7
	G	0	AN ²	0	AN ²	6	AN	4
	H	0	AN ²	0	AN ²	5	AN	5
						Mean:		5.4

¹ Observations: AN = appear normal.² Fungal growth present in this test compartment.³ MAD = missing and assumed dead.

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APPENDIX 7 (continued)

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Observations of Mortality and Effects by Replicate in a 28-Day Sediment Toxicity Test with the Amphipod *Hyalella azteca*

Nominal Concentration (mg/Kg)	Replicate	Day 26		Day 27		No. Dead or MAD ³	Day 28	
		No. Dead	Obs. ¹	No. Dead	Obs.		Obs.	No. Survivors
250	A	0	AN ²	0	AN ²	2	AN	8
	B	0	AN ²	0	AN ²	5	AN	5
	C	0	AN ²	0	AN ²	2	AN	8
	D	0	AN ²	0	AN ²	0	AN	10
	E	0	AN ²	0	AN ²	3	AN	7
	F	0	AN ²	0	AN ²	2	AN	8
	G	0	AN ²	0	AN ²	2	AN	8
	H	0	AN ²	0	AN ²	9	AN	1
						Mean:		6.9
500	A	0	AN ²	0	AN ²	3	AN	7
	B	0	AN ²	0	AN ²	3	AN	7
	C	0	AN ²	0	AN ²	2	AN	8
	D	0	AN ²	0	AN ²	3	AN	7
	E	0	AN ²	0	AN ²	2	AN	8
	F	0	AN ²	0	AN ²	6	AN	4
	G	0	AN ²	0	AN ²	0	AN	10
	H	0	AN ²	0	AN ²	3	AN	7
						Mean:		7.3

¹ Observations: AN = appear normal.² Fungal growth present in this test compartment.³ MAD = missing and assumed dead.

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APPENDIX 7 (continued)

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Observations of Mortality and Effects by Replicate in a 28-Day Sediment Toxicity Test with the Amphipod *Hyalella azteca*

Nominal Concentration (mg/Kg)	Replicate	Day 26		Day 27		Day 28	
		No. Dead	Obs. ¹	No. Dead	Obs.	No. Dead or MAD ³	No. Survivors
1000	A	0	AN ²	0	AN ²	6	AN
	B	0	AN ²	0	AN ²	2	AN
	C	0	AN ²	0	AN ²	1	AN
	D	0	AN ²	0	AN ²	5	AN
	E	0	AN ²	0	AN ²	3	AN
	F	0	AN ²	0	AN ²	4	AN
	G	0	AN ²	0	AN ²	6	AN
	H	0	AN ²	0	AN ²	7	AN
						Mean:	5.8

¹ Observations: AN = appear normal.² Fungal growth present in this test compartment.³ MAD = missing and assumed dead.

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APPENDIX 8

Amphipod Dry Weights by Replicate in a 28-Day Sediment
Toxicity Test with the Amphipod *Hyaella azteca*

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APPENDIX 8

Amphipod Dry Weights by Replicate in a 28-Day Sediment Toxicity Test with the Amphipod *Hyaletella azteca*

Replicate	Average Individual Dry Weight (mg) by Nominal Concentration					
	Negative Control	31 (mg/Kg)	63 (mg/Kg)	125 (mg/Kg)	250 (mg/Kg)	500 (mg/Kg)
A	0.12	0.05	0.11	0.17	0.15	0.13
B	0.15	0.11	0.10	0.12	0.06	0.28
C	0.07	0.07	0.06	0.06	0.12	0.05
D	0.11	0.06	0.19	0.29	0.09	0.15
E	0.07	0.07	0.20	0.17	0.13	0.10
F	0.08	0.24	0.26	0.09	0.11	0.39
G	0.14	0.16	0.07	0.09	0.11	0.09
H	0.14	0.07	0.08	0.15	0.17	0.19
Mean ± Std. Dev.	0.11 ± 0.03	0.10 ± 0.07	0.13 ± 0.07	0.14 ± 0.07	0.12 ± 0.03	0.17 ± 0.12
						0.14 ± 0.05

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APPENDIX 9

HBCD Non-GLP Rangefinding Results

APPENDIX 9

**HBCD Non-GLP Rangefinding Results
Oligochaete**

2% organic carbon

SURVIVAL			
mg/kg	Rep A n=10	Rep B n=10	Total
NC	13	20	33
50	27	39	66
100	33	23	56
500	21	28	49
1000	31	21	52

439A-117

Mean Individual Dry Weight			
mg/kg	Rep A n=10	Rep B n=10	Mean
NC	2.24	1.61	1.925
50	1.88	1.53	1.705
100	1.05	1.48	1.265
500	1.59	1.15	1.37
1000	1.75	2.08	1.915

5% organic carbon

SURVIVAL			
mg/kg	Rep A n=10	Rep B n=10	Total
NC	34	35	69
50	23	24	47
100	33	32	65
500	23	24	47
1000	23	27	50

439A-118

Mean Individual Dry Weight			
mg/kg	Rep A n=10	Rep B n=10	Mean
NC	1.39	0.88	1.135
50	1.25	1.47	1.36
100	1.19	1.33	1.26
500	1.23	1.11	1.17
1000	1.87	1.02	1.445

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APPENDIX 9 (continued)**HBCD Non-GLP Rangefinding Results
Chironomid****2% organic carbon****439A-121**

mg/kg	Rep A n=20	Rep B n=20	Total	% Emergence
NC	19	18	37	92.5
50	8	16	24	60
100	20	18	38	95
500	14	19	33	82.5
1000	20	14	34	85

5% organic carbon**439A-122**

mg/kg	Rep A n=20	Rep B n=20	Total	% Emergence
NC	10	13	23	57.5
50	20	20	40	100
100	16	10	26	65
500	20	15	35	87.5
1000	6	20	26	65

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APPENDIX 9 (continued)**HBCD Non-GLP Rangefinding Results
Hyaella**

2% organic carbon		439A-119
mg/kg	Rep A n=10	% Survival
NC	8	80
50	8	80
100	9	90
500	6	60
1000	4	40

5% organic carbon		439A-120
mg/kg	Rep A n=10	% Survival
NC	9	90
50	8	80
100	8	80
500	4	40
1000	1	10

APPENDIX 10

Personnel Involved in the Study

The following key Wildlife International, Ltd. personnel were involved in the conduct or management of this study:

1. Mark Jaber, Director of Research
2. Henry O. Krueger, Ph.D., Director of Aquatic Toxicology/Terrestrial Plants and Insects
3. Willard B. Nixon, Ph.D., Director of Chemistry
4. Timothy Z. Kendall, Supervisor
5. Cary A. Sutherland, Laboratory Supervisor
6. Susan Thomas, Biologist
7. Tim Ross, Biologist
8. Amy Blankinship, Biologist
9. Michele Stence, Biologist
10. Kathy Dawson, Biologist
11. Debbie Desjardins, Biologist

HEXABROMOCYCLODODECANE (HBCD):
A PROLONGED SEDIMENT TOXICITY TEST WITH
Hyaella azteca USING SPIKED SEDIMENT
WITH 5% TOTAL ORGANIC CARBON

FINAL REPORT

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 439A-120

ASTM E 1706-95b Guideline

U. S. Environmental Protection Agency
Series 850 – Ecological Effects Test Guidelines
OPPTS Number 850.1735

AUTHORS:

Susan Thomas
Henry O. Krueger, Ph.D.
Timothy Z. Kendall

STUDY INITIATION DATE: February 5, 2002

STUDY COMPLETION DATE: June 17, 2003

SUBMITTED TO:

American Chemistry Council's
Brominated Flame Retardant Industry Panel
1300 Wilson Boulevard
Arlington, Virginia 22209

Wildlife International, Ltd.

8598 Commerce Drive
Easton, Maryland 21601
(410) 822-8600

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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

SPONSOR: American Chemistry Council's Brominated Flame Retardant Industry Panel

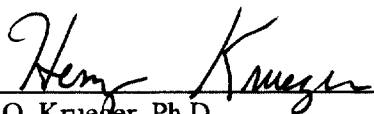
TITLE: Hexabromocyclododecane (HBCD): A Prolonged Sediment Toxicity Test with
Hyalella azteca Using Spiked Sediment with 5% Total Organic Carbon

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 439A-120

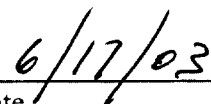
STUDY COMPLETION: June 17, 2003

This study was conducted in compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency in 40 CFR Parts 160 and 792, 17 August 1989; OECD Principles of Good Laboratory Practice (ENV/MC/CHEM (98) 17); and Japan MAFF (59 NohSan, Notification No. 3850, Agricultural Production Bureau), 10 August 1984.

STUDY DIRECTOR:



Henry O. Krueger, Ph.D.
Director of Aquatic Toxicology/ Terrestrial Plants and Insects



Date

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QUALITY ASSURANCE STATEMENT

This study was examined for compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency in 40 CFR Parts 160 and 792, 17 August 1989; OECD Principles of Good Laboratory Practice (ENV/MC/CHEM (98) 17); and Japan MAFF (59 NohSan, Notification No. 3850, Agricultural Production Bureau), 10 August 1984. The dates of all inspections and audits and the dates that any findings were reported to the Study Director and Laboratory Management were as follows:

ACTIVITY:	DATE CONDUCTED:	DATE REPORTED TO:	
		STUDY DIRECTOR:	MANAGEMENT:
Test Substance Preparation	January 17, 2003	January 17, 2003	January 22, 2003
Water Chemistry	February 12, 2003	February 12, 2003	February 20, 2003
Calibration Standard Preparation	February 19, 2003	February 19, 2003	March 25, 2003
Growth Measurements	February 21, 2003	February 21, 2003	February 26, 2003
Analytical Data and Draft Report	April 7 and 8, 2003	April 8, 2003	April 9, 2003
Biology Data and Draft Report	April 16, 17 and 21, 2003	April 21, 2003	May 2, 2003
Final Report	June 12, 2003	June 12, 2003	June 17, 2003

James H. Coleman
James H. Coleman
Quality Assurance Representative

6-17-03
Date

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REPORT APPROVAL

SPONSOR: American Chemistry Council's Brominated Flame Retardant Industry Panel

TITLE: Hexabromocyclododecane (HBCD): A Prolonged Sediment Toxicity Test with
Hyaella azteca Using Spiked Sediment with 5% Total Organic Carbon

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 439A-120

STUDY DIRECTOR:



Henry O. Krueger, Ph.D.
Director of Aquatic Toxicology/ Terrestrial Plants and Insects

6/17/03
Date

PRINCIPAL INVESTIGATOR:



Timothy Z. Kendall
Laboratory Supervisor

6/17/03
Date

MANAGEMENT:



Willard B. Nixon, Ph.D.
Director of Chemistry

6/17/03
Date

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SUMMARY

SPONSOR:	American Chemistry Council's Brominated Flame Retardant Industry Panel
SPONSOR'S REPRESENTATIVE:	Wendy Sherman
LOCATION OF STUDY, RAW DATA AND A COPY OF THE FINAL REPORT:	Wildlife International, Ltd. Easton, Maryland 21601

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER:	439A-120
TEST SUBSTANCE:	Hexabromocyclododecane (HBCD)
STUDY:	Hexabromocyclododecane (HBCD): A Prolonged Sediment Toxicity Test with <i>Hyalella azteca</i> Using Spiked Sediment with 5% Total Organic Carbon
NOMINAL SEDIMENT CONCENTRATIONS:	Negative Control, 31, 63, 125, 250, 500 and 1000 mg/Kg dry weight of sediment
TEST DATES:	Experimental Start (OECD) – January 17, 2003 Experimental Start (EPA) – January 22, 2003 Biological Termination – February 19, 2003 Analytical Termination – February 19, 2003 Experimental Termination – February 21, 2003
LENGTH OF EXPOSURE:	28 Days

TEST ORGANISM:	Amphipod (<i>Hyalella azteca</i>)
SOURCE OF TEST ORGANISMS:	Environmental Consulting and Testing 1423 N. 8 th Street Suite 118 Superior, Wisconsin 54880
AGE OF TEST ORGANISM:	13 days

28 DAY EC50:	> 1000 mg/Kg dry weight of sediment
95% CONFIDENCE LIMITS:	Not calculable
LOWEST-OBSERVED-EFFECT-CONCENTRATION:	> 1000 mg/Kg dry weight of sediment
NO-OBSERVED-EFFECT-CONCENTRATION:	1000 mg/Kg dry weight of sediment

INTRODUCTION

This study was conducted by Wildlife International, Ltd. for American Chemistry Council's Brominated Flame Retardant Industry Panel at the Wildlife International, Ltd. aquatic toxicology facility in Easton, Maryland. The in-life phase of the test was conducted from January 22, 2003 to February 19, 2003. Raw data generated by Wildlife International, Ltd. and a copy of the final report are filed under Project Number 439A-120 in archives located on the Wildlife International, Ltd. site.

OBJECTIVE

The objective of this study was to determine the effects of sediment-incorporated hexabromocyclododecane, also known as HBCD, on the amphipod, *Hyalella azteca*, during a 28-day exposure period under flow-through test conditions. The measured endpoints of the test were survivorship and growth as determined by dry weight measurements.

EXPERIMENTAL DESIGN

Non-GLP exploratory range-finding studies were performed with three freshwater species associated with sediment: oligochaetes (*Lumbriculus variegatus*), chironomids (*Chironomus riparius*) and amphipods (*Hyalella azteca*). All three species were tested at 50, 100, 500, 1000 mg/Kg dry sediment in two different types sediments, one with a 2% organic carbon content and the other with a 5% organic carbon content. Based on the results of the range-finding studies (Appendix 9), the amphipods were found to be the most sensitive species in both sediment types, with clear effects in the 500 mg/Kg treatment group. Therefore, more definitive GLP studies were conducted with the freshwater amphipod, *Hyalella azteca* in both sediment types. This study reports the results of HBCD exposure in the 5% organic content sediment.

Groups of amphipods were exposed to a geometric series of six test concentrations and a negative control for 28 days under flow-through test conditions. Eight replicate test compartments were maintained in each treatment and control group, with 10 amphipods in each test compartment, for a total of 80 amphipods per test concentration. Each test compartment contained a quantity of sediment and overlying water. Additional replicates were added in the control group, low and high treatment groups for analytical sampling of water and sediment. The "analytical" replicates sampled on Day 0 contained

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no amphipods, while amphipods were added at test initiation to the "analytical" replicates sampled on Day 7 and at test termination.

Nominal test concentrations selected in consultation with the sponsor were 31, 63, 125, 250, 500 and 1000 mg/Kg of sediment based on the dry weight of the sediment. The results of the study are based on the nominal test concentrations. Overlying water, pore water and sediment samples were collected and analyzed from the "analytical replicates" of the control group and the lowest and highest test concentrations. The collection and analysis were done approximately ten minutes after the addition of test organisms to the test system on Day 0, on Day 7 and at the end of the test. Results of the analyses were used to confirm the lowest and highest test concentrations.

Test compartments were impartially positioned in a diluter unit approximately 2 days prior to test initiation to condition the sediment prior to introduction of organisms. Amphipods were impartially assigned to exposure compartments at test initiation. Observations of mortality and abnormal behavior were made at least three times per week during the test. Survivorship and growth (dry weights) were determined at the end of the 28-day test period. The percent reduction in the numbers of organisms present in the treatment groups at test termination in comparison to the control group was used to determine the 28-day EC50 value. The lowest-observed-effect-concentration (LOEC) and the no-observed-effect-concentration (NOEC) were determined by the concentration-response pattern and statistical analysis of the survival and dry weight data.

MATERIALS AND METHODS

The study was conducted based on the procedures outlined in the protocol, "Hexabromocyclododecane (HBCD): A Prolonged Sediment Toxicity Test with *Hyalella azteca* Using Spiked Sediment with 5% Total Organic Carbon" (Appendix I). The protocol was based on the ASTM E 1706-95b Guideline: Standard Test Methods for Measuring the Toxicity of Sediment-Associated Contaminants with Fresh Water Invertebrates (1); the OPPTS 850.1735 Guideline: Whole Sediment Acute Toxicity Invertebrates, Freshwater (2); and Methods for Measuring the Toxicity and Bioaccumulation of Sediment-Associated Contaminants with Freshwater Invertebrates (2nd ed.) (3).

Test Substance

The test substance consisted of a composite of hexabromocyclododecane (CAS No. 25637-99-4) samples received from three manufacturers. The material's identity and date received from each of the manufacturers is given below:

<u>Manufacturer</u>	<u>Lot/Batch</u>	<u>Date Received</u>	Wildlife International, Ltd. <u>Identification Number</u>
Albemarle Corporation	33449-15X	December 20, 1995	3519
Great Lakes Chemical Corporation	Not Given	June 19, 1998	4515
Eurobrom b.v.	010328	December 10, 2001	5827

The composite test substance was assigned Wildlife International, Ltd. identification number 5850 and was stored under ambient conditions. The composite test substance was shipped to Albemarle Corporation for characterization and purity analyses (Appendix 2). The results of the analyses indicated the composite test substance was homogeneous, and the test substance was assigned an expiration date of February 21, 2003.

Test Sediment

Artificial sediment described as "Formulated Sediment A" in Kemble, et al. (4) was used as the test sediment. This artificial sediment is similar to that described in OECD Guideline 207 (5), but uses alpha-cellulose as its source of organic matter instead of peat moss. Alpha-cellulose was selected by Kemble, et al. (4) as a more standardized source of organic matter than peat moss.

The soil was composed of approximately 0.01% humic acid, 0.5% dolomite, 13% alpha-cellulose, 10% kaolin clay, and 77% industrial quartz sand (Appendix 3). The dry constituents of the soil were mixed in a PK-Twinshell mixer for 20 minutes. The dry soil was stored under ambient conditions until used. The soil was characterized (Agvise Laboratories, Northwood, North Dakota) as having a mean organic matter content of 8.2%, a mean organic carbon content of 4.7%, a water holding capacity of 11.9%, pH of 8.2, and particle distribution of 85% sand, 8% silt, and 7% clay.

Preparation of Test Concentrations

Concentrations of the test substance in the sediment were prepared on a dry weight basis (i.e., mg test substance/Kg dry sediment). The test substance was weighed on tared weigh paper then added directly to the sediment. The sediment and test substance were mixed for approximately 22 hours on a rotating mixer.

Eight replicate test compartments were prepared for each treatment and control group. Six additional compartments were prepared for the control, low and high treatment groups, for analytical purposes. For each replicate, 100 mL of sediment was placed in a 300-mL glass beaker. The prepared test compartments were indiscriminately and slowly positioned in diluter tanks according to treatment groups and the overlying water (approximately 100-150 ml) was allowed to flow through the test compartments. The sediment/water mixtures were allowed to acclimate for approximately 2 days prior to the introduction of the test organisms. At test initiation and termination, the overlying water in all test chambers appeared clear and colorless but fungal growth was present at test termination.

Test Organism

The amphipod, *Hyaella azteca*, was selected as the test species for this study. Amphipods are representative of an important group of aquatic invertebrates and were selected for use in the study based upon past history of use and ease of culturing in the laboratory. The organisms were obtained from Environmental Consulting and Testing, Superior, Wisconsin. The identity of the species was verified by the supplier.

The organisms were held in water from the same source as the water used during the test. During the 5-day holding period preceding the test, water temperatures ranged from 23.2 to 24.0°C, measured with a hand-held liquid-in-glass thermometer. The pH of the water ranged from 8.5 to 8.9, measured with a Fisher Scientific Accumet Model 915 pH meter. Dissolved oxygen ranged from 8.0 to 8.3 mg/L (>93% of saturation), measured with a Yellow Springs Instruments Model 51B dissolved oxygen meter.

During holding, the amphipods appeared normal. At test initiation, amphipods from the culture were impartially added one and two at a time to each test compartment. All transfers were made below the water surface using wide-bore pipettes. Eight replicate test chambers in each treatment and control

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group contained organisms. Of the additional six analytical replicates in the control, low and high treatment groups, the two replicates sampled on Day 0 did not contain organisms, while the test chambers sampled on Day 7 and 28 did contain organisms.

Amphipods were fed daily a mixture of yeast, Cerophyll[®] and trout chow (YCT) and *Selenastrum* during the holding period, but only YCT during the test. A 1.0 mL aliquot of food was added to each test compartment daily throughout the test except on days 6, 12, 14 and 16. On day 6, the food was reduced to 0.5 mL due to a lack of availability of food. On days 12, 14 and 16, the food was withheld due to fungal growth.

Dilution Water

The water used for culturing and testing was freshwater obtained from a well approximately 40 meters deep located on the Wildlife International, Ltd. site. The well water is characterized as moderately-hard water. The specific conductance, hardness, alkalinity and pH measurements of the well water during the four-week period immediately preceding the test are presented in Appendix IV.

The well water was passed through a sand filter to remove particles greater than approximately 25 μm , and pumped into a 37,800-L storage tank where the water was aerated with spray nozzles. Prior to use, the water again was filtered (0.45 μm) to remove microorganisms and particles. The results of periodic analyses performed to measure the concentrations of selected organic and inorganic constituents in the well water used by Wildlife International, Ltd. are presented in Appendix 5.

Test Apparatus

The test apparatus consisted of a Wildlife International, Ltd. diluter unit that held seven 52-L stainless steel diluter tanks. Siphoning systems were placed in each tank to vary the water height, allowing the dilution water to flow through the test compartments. The depth of water in one representative test compartment was 4.3 cm. The diluter was adjusted so that each test chamber received two or less volume additions of water per day.

Test compartments were 300-mL glass beakers with two stainless steel mesh-covered holes on opposite sides to allow for the flow of water through the test compartment. Each beaker contained approximately 100 mL of sediment and approximately 100 to 150 mL of overlying water. The depth of

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sediment in one representative test compartment was 3.9 cm. The depth of overlying water in each test compartment was maintained by the water levels in the diluter tanks. Test compartments were covered with plastic petri dishes and were labeled with the project number, test concentration and replicate.

Environmental Conditions

Lighting used to illuminate the culture and test chambers during holding and testing was provided by fluorescent tubes that emitted wavelengths similar to natural sunlight (Colortone® 50). A photoperiod of 16 hours of light and 8 hours of darkness was controlled with an automatic timer. A 30-minute transition period of low light intensity was provided when lights went on and off to avoid sudden changes in light intensity. Light intensity at test initiation was 219 lux at the surface of the water over one representative test chamber.

The target test temperature during the study was $23 \pm 2^{\circ}\text{C}$. Temperature was measured in the overlying water of one alternating replicate test compartment at the beginning of the test, three times per week during the test, and at the end of the test using a hand-held liquid-in-glass thermometer. Temperature also was measured continuously in the negative control diluter tank using a Fulscope ER/C Recorder. The continuous recorder was verified with a hand-held liquid-in-glass thermometer prior to test initiation and at least weekly during the test.

Hardness, alkalinity, specific conductance and ammonia measurements were made on samples of overlying water collected from one replicate test compartment of each treatment and control group at test initiation and termination. Dissolved oxygen measurements were made on samples of overlying water collected from one alternating replicate test compartment of each treatment and control group at test initiation, three times per week during the test, and at test termination. On Day 21, the dissolved oxygen levels fell below 60% saturation in the 125 and 500 mg/Kg treatment groups. Dissolved oxygen measurements were performed in every replicate of those treatment groups on that day. After consultation with the Sponsor, aeration was introduced to the test chambers on Day 22 and continued for the rest of the test. Measurements of pH were made on samples of overlying water collected from one alternating replicate test compartment of each treatment and control group at test initiation, once each week during the test, and at test termination.

Light intensity was measured using a SPER Scientific Model 840006C light meter. Dissolved oxygen was measured using a Thermo Orion model 850Aplus dissolved oxygen meter, and

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measurements of pH were made using a Thermo Orion model 525Aplus meter. Hardness and Alkalinity measurements were made by titration using procedures based on methods in *Standard Methods for the Examination of Water and Wastewater* (6). Specific Conductance was measured using a Yellow Springs Instrument Model 33 Salinity-Conductivity-Temperature meter. Ammonia was measured using a Thermo Orion Model 720Aplus pH/ISE meter.

Observations

The test chambers were observed at least three times per week to determine the number of mortalities and the number of individuals exhibiting signs of toxicity or abnormal behavior. On Day 28 of the test, amphipods were segregated from the sediment, and the numbers of live or dead amphipods were enumerated.

Dry Weight Measurements

At test termination, surviving amphipods were rinsed of excess sediment, placed in tared crucibles by replicate and dried for approximately 41.5 hours at approximately 60°C. The amphipods were weighed after allowing the crucibles to cool at room temperature for at least one hour.

Statistical Analyses

The results of the test were based on the Day 0 nominal sediment concentrations. Since the percent reduction in the number of organisms present at test termination in comparison to the negative control group was less than 50% in all treatment groups, the 28-day EC50 value was estimated to be greater than 1000 mg/Kg of dry sediment, the highest concentration tested.

The no-observed-effect-concentration (NOEC) and lowest-observed-effect-concentration (LOEC) were determined by visual interpretation of the dose-response pattern and statistical analyses of the survival and dry weight data. The survival and dry weight (growth) data were evaluated for normality (Chi-Square Test) and homogeneity of variances (Bartlett's Test). After the data was deemed normal with homogeneous variance, the survival and dry weight data were analyzed using Fisher's Exact test and Bonferroni t-Test to identify those treatment levels that were statistically different ($p < 0.05$) from the negative control group (7, 8).

Collection of Analytical Samples

Analyses of sediment and water were conducted using separate test chambers collected from the control group and the lowest and highest concentration treatment groups approximately 10 minutes after the introduction of the organisms on Day 0, on Day 7 and at test termination on Day 28. Samples of overlying water, pore water and sediment were collected from each of the test chambers at each sampling interval. The replicates used for sample collection on Day 0 did not contain amphipods, whereas at test initiation amphipods were placed in the replicates used for sample collection on Days 7 and 28. Samples of the overlying water were collected at mid-depth in the water column, and then the remaining overlying water was poured from each test chamber. The remaining sediment was collected, centrifuged, and split into separate samples of pore water and sediment. The samples were processed for analysis immediately on the day of collection, and analyzed within one day.

Analytical Methods

Concentrations of HBCD in sediment and aqueous solution samples were determined by high performance liquid chromatography using an Agilent Model 1100 High Performance Liquid Chromatograph (HPLC) equipped with an Agilent Series 1100 Variable Wavelength Detector. Chromatographic separations were achieved using a YMC-PACK ODS-AM analytical column (150 mm x 4.6 mm, 3 μ m particle size). The instrument parameters are summarized in Appendix 6.1.

The analytical methods used for the analysis of Hexabromocyclododecane (HBCD) in sediment, pore water and aqueous solutions were developed by Wildlife International, Ltd. Samples were processed as follows:

All sediment samples were prepared by weighing 2.0 g of soil in scintillation vials. Samples were sonic disrupted for approximately 10 minutes after adding 10.0 mL of Tetrahydrofuran. Samples were then centrifuged for approximately 15 minutes at approximately 1500 rpm and extract was transferred to a roundbottom flask. The extraction was repeated with an additional 10 mL of THF and centrifuged extract was added to the appropriate roundbottom flask. Each sample was rotary evaporated to approximately 0.50 – 1.0 mL using a waterbath maintained at approximately 40°C. The samples were then evaporated to dryness under a gentle stream of nitrogen. The requisite volume of THF was volumetrically added to each roundbottom flask and swirled well. Secondary dilutions in 50% THF : 50% water were performed. Aliquots of each reconstituted sample were transferred to

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autosampler vials and submitted for HPLC/UV analysis. A method flow chart is provided in Appendix 6.2.

For aqueous solutions, QC samples were prepared by adding 50 mL of well water to a 125-mL separatory funnel and fortifying with the appropriate HBCD stock solution. Dichloromethane (DCM) (25 mL) was added to each separatory funnel. From each test solution, 50 mL was transferred directly into a separatory funnel containing 25 mL of dichloromethane. The QC and study sample solutions were shaken (with venting) for approximately one minute and the two phases were allowed to separate. The lower organic layer was drained into a 125-mL roundbottom flask and a second 25-mL aliquot of DCM was added to the aqueous phase remaining in the funnel to perform a second extraction. The organic phase from the second extraction was combined with the organic phase from the first extraction in the same roundbottom flask. The organic extracts were rotary evaporated to 1-2 mL under vacuum and a waterbath. The residue DCM in each flask was evaporated to dryness under a gentle stream of nitrogen. The residues were reconstituted with an appropriate volume of 50% THF : 50% water. Aliquots of the diluted extracts were transferred into an autosampler vial and submitted for HPLC/UV analysis. A method flow chart is provided in Appendix 6.13.

Calibration Curves

Five calibration standards of HBCD, ranging in concentration from 1.00 to 10.0 mg/L (Appendix 6.3) for both sediment and freshwater were analyzed with the sample sets. The calibration standard series for each analysis was injected at the beginning and end of each analytical run. In addition, a minimum of one calibration standard was analyzed after every five test samples. Linear regression equations were generated using the peak area responses versus the respective concentrations of the calibration standards. The concentration of HBCD in the samples was determined by substituting the peak area response into the applicable linear regression equation. An example of the calculations for a representative sample is included in Appendix 6.4.

Limits of Quantitation (LOQ)

The limit of quantitation (LOQ) for the overlying samples was defined as 0.100 mg/L, calculated as the product of the lowest calibration standard analyzed (1.00 mg/L) and the dilution factor of the matrix blank samples (0.100). The limits of quantitation (LOQ) for the pore water samples were individually calculated as the product of the concentration of the lowest calibration

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standard analyzed (1.00 mg/L) and the dilution factor of each test sample (0.100). The limit of quantitation (LOQ) for the sediment samples was defined as 12.5 µg/g, calculated as the product of the lowest calibration standard concentration analyzed (1.00 mg/L) and the dilution factor of the matrix blank samples (12.5).

Matrix Blank and Fortification Samples

One matrix blank sample for sediment was analyzed at each interval to determine possible interferences. No interferences were observed at or above the LOQ during the sample analyses (Appendix 6.5).

Sediment samples were fortified at 30.0 and 1000 µg/g using a stock solution of HBCD prepared in tetrahydrofuran (Appendix 6.3) and were analyzed concurrently with the samples. The measured concentrations for the matrix fortification samples in sediment ranged from 98.5 to 102% of nominal concentrations (Appendix 6.5).

One matrix blank sample for water was analyzed at each interval to determine possible interferences. No interferences were observed at or above the LOQ during the Days 0, 7 and 28 sample analyses (Appendix 6.6).

Samples of freshwater were fortified at 0.200 and 0.500 mg/L using stock solutions of HBCD prepared in tetrahydrofuran (Appendix 6.3), and were analyzed concurrently with the samples. The measured concentrations for the matrix fortification samples in freshwater ranged from 66.7 to 103% of nominal concentrations (Appendix 6.6).

A representative calibration curve for sediment is presented in Appendix 6.7. Representative chromatograms of low and high-level calibration standards for sediment are presented in Appendices 6.8 and 6.9, respectively. Representative chromatograms of a matrix blank sample and a matrix fortification sample are presented in Appendices 6.10 and 6.11, respectively. A representative chromatogram of a Day 0 sediment sample is presented in Appendix 6.12.

A representative calibration curve for freshwater is presented in Appendix 6.14. Representative chromatograms of low and high-level calibration standards for freshwater are presented in

Appendices 6.15 and 6.16, respectively. Representative chromatograms of a matrix blank sample and a matrix fortification sample are presented in Appendices 6.17 and 6.18, respectively. Representative chromatograms of a Day 0 overlying water sample and a Day 0 pore water sample are presented in Appendices 6.19 and 6.20, respectively.

RESULTS AND DISCUSSION

Confirmation of Test Concentrations

Results of analyses to measure concentrations of hexabromocyclododecane (HBCD) in the sediment, pore water and overlying water samples collected during the definitive test are presented in Tables 1, 2, and 3, respectively. Samples from the low and high test concentrations confirmed that the HBCD in sediment tended to remain in sediment and not move into the pore water or overlying water. The results from sediment samples from the highest test concentration (1000 mg/Kg) ranged between 122 and 78.2% of nominal, while values from the lowest concentration (31 mg/Kg) were more variable ranging from below the LOQ (12.5 mg/Kg) to 197% of nominal. All samples of overlying water were below the limit of quantitation. Pore water samples from the low concentration were also below the limit of quantitation, but pore water samples from the highest test concentration had values in the low ppm range. These values are well above solubility and were believed to be the result of small particles of HBCD being extracted out of the pore water, artificially inflating the reported values.

Observations and Measurements

Measurements of temperature, dissolved oxygen and pH of the overlying water in the test chambers are presented in Tables 4, 5 and 6, respectively. Temperatures were within the $23 \pm 2^{\circ}\text{C}$ range established for the test. Dissolved oxygen concentrations were $\geq 49\%$ (4.2 mg/L) of saturation throughout the test. From Day 22 to test termination, gentle aeration was introduced to the test chambers to improve the dissolved oxygen concentration in the water overlying the sediment. Measurements of pH ranged from 7.8 to 8.4 during the test. Hardness values ranged from 136 to 144 mg/L as CaCO_3 . Alkalinity values ranged from 184 to 188 mg/L as CaCO_3 during the test. Conductivity ranged from 310 to 330 $\mu\text{mhos/cm}$ during the test. Ammonia ranged from 0.082 to 1.2 mg/L as NH_3 during the test.

Observations of amphipods in individual replicates at each observation interval are presented in Appendix 7. All replicates observed during the test appeared normal, with some direct observations of

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mortality in the 31, 63, 125, 250 and 500 mg/Kg treatment groups and the control during the test. The presence of fungal growth was noted in all replicates, in all treatment groups and the control during the test.

The mean number of amphipods in the negative control, 31, 63, 125, 250, 500 and 1000 mg/Kg treatment groups at test termination was 9.1, 8.6, 5.9, 6.1, 7.0, 8.5 and 9.1, respectively (Table 8). The mean numbers of amphipods in the 63, 125 and 250 mg/Kg treatment groups were found to be statistically different ($p \leq 0.05$) from the negative control group. Survival in the 31, 500 and 1000 mg/Kg treatment groups was similar to the control group and any differences were not statistically significant ($p > 0.05$). Since the percent reduction in the number of organisms present at test termination in comparison to the negative control group was less than 50% in all treatment groups (Table 8), the 28-day EC50 value was estimated to be greater than 1000 mg/Kg of dry sediment, the highest concentration tested. The percent reduction from the control in the 63, 125, and 250 mg/Kg treatment groups was 35.2, 33.0, and 23.1%, respectively. The mortality in these groups was moderate and there were clearly no effects at the 500 and 1000 mg/Kg treatment levels. Therefore, the mortality observed in the middle test concentrations was not considered treatment related since there was no evidence of a concentration dependent response.

The average individual dry weights for the surviving amphipods in each replicate at test termination are presented in Appendix 8. The average dry weight per amphipod in the negative control group was 0.19 mg (Table 8). The average dry weight per amphipod in the 31, 63, 125, 250, 500 and 1000 mg/Kg treatment groups was 0.17, 0.26, 0.22, 0.20, 0.19 and 0.19 mg, respectively. The dry weights were not significantly different ($p > 0.05$) from the negative control weights, and any differences were not concentration-dependent. Therefore, there were no apparent effects on growth (dry weight) observed at test termination.

CONCLUSIONS

The 28-day EC50 value for amphipods (*Hyalella azteca*) exposed to hexabromocyclododecane in sediment was >1000 mg/Kg dry weight of sediment, the highest nominal concentration tested. Determination of the lowest-observed-effect-concentration (LOEC) and the no-observed-effect-concentration (NOEC) was based on an evaluation of the survival and growth (dry weight) data. The

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most sensitive parameter in this study was survival. Based on the results of this study, the LOEC was >1000 mg/Kg dry weight of sediment and the NOEC was 1000 mg/Kg dry weight of sediment.

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Table 1

Measured Concentrations of Hexabromocyclododecane (HBCD) in Sediment Samples
from a Survival and Growth Sediment Toxicity Test with *Hyalella Azteca*

Nominal Sediment Concentration (µg/g)	Sample Number (439A-120-)	Sampling Interval (Day)	Measured HBCD Concentration (µg/g) ^{1,2}	Percent of Nominal ³
0.0 (Negative Control)	S-1	0	< LOQ	--
	S-2	0	< LOQ	--
	S-7	7	< LOQ	--
	S-8	7	< LOQ	--
	S-13	28	< LOQ	--
	S-14	28	< LOQ	--
31	S-3	0	< LOQ	--
	S-4	0	39.1	126
	S-9	7	42.8	138
	S-10	7	26.6	85.7
	S-15	28	45.4	147
	S-16	28	61.0	197
1000	S-5	0	1219	122
	S-6	0	1134	113
	S-11	7	982	98.2
	S-12	7	795	79.5
	S-17	28	782	78.2
	S-18	28	832	83.2

¹ The limit of quantitation (LOQ) was 12.5 µg/g calculated as the product of the concentration of the lowest calibration standard (1.00 mg/L) and the dilution factor of the matrix blank samples (12.5).

² Analytical results were generated using wet weights. The tabulated values are reported on a dry weight basis.

³ Results were generated using Excel 2000 in the full precision mode. Manual calculations may differ slightly.

Table 2

Measured Concentrations of Hexabromocyclododecane (HBCD) in Overlying Water Samples
from a Survival and Growth Sediment Toxicity Test with *Hyalella Azteca*

Nominal Test Concentration ($\mu\text{g/g}$) ¹	Sample Number (439A-120-)	Sampling Interval (Day)	Measured HBCD Concentration (mg/L) ^{2,3}
0.0 (Negative Control)	W-1	0	< LOQ
	W-2	0	< LOQ
	W-7	7	< LOQ
	W-8	7	< LOQ
	W-13	28	< LOQ
	W-14	28	< LOQ
31	W-3	0	< LOQ
	W-4	0	< LOQ
	W-9	7	< LOQ
	W-10	7	< LOQ
	W-15	28	< LOQ
	W-16	28	< LOQ
1000	W-5	0	< LOQ
	W-6	0	< LOQ
	W-11	7	< LOQ
	W-12	7	< LOQ
	W-17	28	< LOQ
	W-18	28	< LOQ

¹ Nominal concentration of sediment samples.

² The limit of quantitation (LOQ) was 0.100 mg/L, calculated as the product of the concentration of the lowest calibration standard (1.00 mg/L) and the dilution factor of the matrix blank samples (0.100).

³ Results were generated using Excel 2000 in the full precision mode. Manual calculations may differ slightly.

Table 3

Measured Concentrations of Hexabromocyclododecane (HBCD) in Pore Water Samples
from a Survival and Growth Sediment Toxicity Test with *Hyaella Azteca*

Nominal Test Concentration ($\mu\text{g/g}$)	Sample Number (439A-120-)	Sampling Interval (Day)	Measured HBCD Concentration (mg/L) ^{1,2}
0.0 (Negative Control)	PW-1	0	< 0.400
	PW-2	0	< 0.417
	PW-7	7	< 0.303
	PW-8	7	< 0.294
	PW-13	28	< 0.263
	PW-14	28	< 0.263
31	PW-3	0	< 0.357
	PW-4	0	< 0.417
	PW-9	7	< 0.345
	PW-10	7	< 0.333
	PW-15	28	< 0.313
	PW-16	28	< 0.333
1000	PW-5*	0	7.02
	PW-6*	0	9.61
	PW-11*	7	5.65
	PW-12*	7	5.25
	PW-17	28	2.23
	PW-18	28	2.56

¹ The limits of quantitation (LOQ) for pore water samples were calculated individually as the product of the concentration of the lowest calibration standard (1.00 mg/L) and the dilution factor of each test sample.

² Results were generated using Excel 2000 in the full precision mode. Manual calculations may differ slightly.

*Samples reanalyzed.

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Table 4

Temperature of Overlying Water in the Test Compartments

Nominal Concentration (mg/Kg)	Temperature (°C) ¹															
	Day															
	Replicate	A	B	C	D	E	F	G	H	A	B	C	D	E	F	G
Negative Control		22.5	22.2	22.1	22.3	22.5	22.5	22.7	22.7	22.6	22.6	22.5	22.7	22.7	22.8	22.8
31		22.4	22.1	22.1	22.2	22.5	22.4	22.7	22.7	22.6	22.4	22.3	22.7	22.6	22.8	22.7
63		22.4	22.3	22.2	22.3	22.5	22.4	22.8	22.8	22.6	22.7	22.5	22.8	22.7	23.0	22.8
125		22.3	22.1	22.2	22.3	22.4	22.4	22.7	22.7	22.4	22.4	22.1	22.5	22.6	22.7	22.7
250		22.5	22.3	22.2	22.3	22.9	22.6	22.9	22.9	22.6	22.6	22.6	22.8	22.7	23.0	22.8
500		22.3	22.0	22.1	22.4	22.4	22.3	22.7	22.7	22.5	22.4	22.2	22.6	22.4	22.8	22.7
1000		22.3	22.0	22.0	22.2	22.5	22.3	22.7	22.7	22.5	22.4	22.3	22.6	22.5	22.7	22.6

¹Manual temperature measurements. Temperature measured continuously during the test ranged from approximately 21.0 to 23.0, measured to the nearest 0.5°C.

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Table 5
Dissolved Oxygen in Overlying Water in the Test Compartments

Nominal Concentration (mg/Kg)	Day Replicate	Dissolved Oxygen (mg/L) ¹															
		0	1	2	5	7	9	12	14	16	19	21	23	26	27	28	
		A	B	C	D	E	F	G	H	A	B	C	D	E	F	G	
Negative Control		8.5	8.2	8.0	8.0	8.2	8.0	7.6	7.7	7.1	7.6	6.1	6.4	5.7	8.2	8.1	
31		8.5	7.9	7.8	8.0	7.6	7.9	7.3	7.8	6.8	6.6	5.4	6.4	6.1	8.2	8.0	
63		8.5	8.2	8.1	8.4	8.1	8.3	7.8	7.6	7.9	7.8	7.6	7.2	7.2	8.1	8.3	
125		8.5	7.8	7.6	7.8	8.1	8.4	7.0	7.3	6.9	7.1	4.5 ²	7.7	7.1	8.3	8.2	
250		8.5	8.4	7.9	8.0	8.2	7.8	7.5	7.7	7.7	8.5	7.3	8.2	7.2	8.2	7.6	
500		8.5	8.2	7.8	7.7	8.1	7.5	6.0	7.1	6.7	8.5	4.2 ²	6.6	5.5	8.4	8.1	
1000		8.5	8.2	7.9	8.4	7.9	8.4	6.9	6.8	7.6	7.9	6.8	7.3	7.2	7.4	8.0	

¹A dissolved oxygen concentration of 5.1 mg/L represents 60% saturation at 23.0°C.

²Aeration added to all test chambers on Day 22.

¹A dissolved oxygen concentration of 5.1 mg/L represents 60% saturation at 23.0°C.

²Aeration added to all test chambers on Day 22.

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Table 6

pH of Overlying Water in the Test Compartments

Nominal Concentration (mg/Kg)	Day: Replicate:	pH				
		0	7	14	21	28
		A	E	H	C	G
Negative Control		8.2	8.1	8.1	8.0	8.2
31		8.2	8.1	8.1	8.0	8.2
63		8.2	8.1	8.1	8.1	8.2
125		8.2	8.1	8.1	7.8	8.2
250		8.2	8.2	8.1	8.1	8.3
500		8.2	8.2	8.1	7.9	8.3
1000		8.2	8.2	8.1	8.1	8.4

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Table 7

Hardness, Alkalinity, Conductivity and Ammonia of Overlying Water in Test Compartments

Nominal Concentration (mg/Kg)	Day 0 (Replicate A)				Day 28 (Replicate G)			
	Hardness (mg/L as CaCO ₃)	Alkalinity (mg/L as CaCO ₃)	Conductivity (µmhos/cm)	Ammonia (mg/L as NH ₃)	Hardness (mg/L as CaCO ₃)	Alkalinity (mg/L as CaCO ₃)	Conductivity (µmhos/cm)	Ammonia (mg/L as NH ₃)
Negative Control	140	184	320	0.54	140	186	310	1.2
31	140	184	330	0.71	140	188	310	0.31
63	136	184	330	0.69	140	188	315	1.0
125	140	184	320	1.1	144	186	320	0.082
250	140	184	330	1.1	140	186	315	0.31
500	140	184	330	0.75	144	186	320	0.16
1000	136	184	330	0.65	144	186	315	0.15

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Table 8

Mean Survival and Growth of Amphipods
(*Hyalella azteca*) During a 28-Day Sediment Toxicity Test

Nominal Concentration (mg/Kg)	Mean Number of Surviving Amphipods ¹	Percent Reduction in Survival ^{2, 3} (%)	Mean Individual Dry Weight ⁴ (mg)	Percent Reduction in Dry Weight ² (%)
Negative Control	9.1	--	0.19	--
31	8.6	5.5	0.17	10.5
63	5.9	35.2	0.26	-36.8
125	6.1	33.0	0.22	-15.8
250	7.0	23.1	0.20	-5.3
500	8.5	6.6	0.19	0
1000	9.1	0	0.19	0

¹ Each replicate contained 10 amphipods at test initiation.

² Percent reduction was calculated in relation to the mean of the negative control.

³ There were statistically significant differences ($p \leq 0.05$) in the mean number of surviving amphipods for the 63, 125 and 250 mg/Kg treatment groups in comparison to the negative control group using Fisher's Exact test; however the differences are not believed to be treatment related.

⁴ There were no statistically significant differences ($p > 0.05$) in the mean individual dry weight in comparison to the negative control group using the Bonferroni t-test.

APPENDIX 1

Protocol, Protocol Amendment and Deviation

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PROTOCOL

HEXABROMOCYCLODODECANE (HBCD): A PROLONGED SEDIMENT TOXICITY
TEST WITH *Hyaella azteca* USING SPIKED SEDIMENT
WITH 5% TOTAL ORGANIC CARBON

ASTM E 1706-95b Guideline

U.S. Environmental Protection Agency
Series 850 - Ecological Effects Test Guidelines
OPPTS Number 850.1735

Submitted to

American Chemistry Council's
Brominated Flame Retardant Industry Panel
1300 Wilson Boulevard
Arlington, Virginia 22209

Wildlife International, Ltd.

8598 Commerce Drive
Easton, Maryland 21601
(410) 822-8600

January 3, 2002

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Wildlife International, Ltd.

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HEXABROMOCYCLODODECANE (HBCD): A PROLONGED SEDIMENT TOXICITY
TEST WITH *Hyalomma azteca* USING SPIKED SEDIMENT
WITH 5% TOTAL ORGANIC CARBON

SPONSOR: American Chemistry Council's
Brominated Flame Retardant Industry Panel
1300 Wilson Boulevard
Arlington, Virginia 22209

SPONSOR'S REPRESENTATIVE: Ms. Wendy Sherman

TESTING FACILITY: Wildlife International, Ltd.
8598 Commerce Drive
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STUDY DIRECTOR: Henry O. Krueger, Ph.D.,
Director of Aquatic Toxicology & Non-Target Plants

LABORATORY MANAGEMENT: ~~Henry O. Krueger, Ph.D.~~ *Will D. Kelly, Ph.D.*
Director of Aquatic Toxicology & Non-Target Plants
Analytical Chemistry *HK 2/5/02*

FOR LABORATORY USE ONLY

Proposed Dates:	
Experimental Start Date: _____	Experimental Termination Date: _____
Project No.: <u>439A-120</u>	
Test Concentrations: _____	
Test Substance No.: <u>5850</u>	

PROTOCOL APPROVAL

H. O. Krueger
STUDY DIRECTOR

2/5/02
DATE

Will D. Kelly
LABORATORY MANAGEMENT

2/7/02
DATE

Wendy K. Sherman
SPONSOR'S REPRESENTATIVE

January 10, 2002
DATE

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Wildlife International, Ltd.

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INTRODUCTION

Wildlife International, Ltd. will conduct a prolonged sediment toxicity test with the amphipod, *Hyalella azteca*, for the Sponsor at the Wildlife International, Ltd. aquatic toxicology facility in Easton, Maryland. The study will be based upon the ASTM E 1706-95b Guideline: Standard Test Methods for Measuring the Toxicity of Sediment-Associated Contaminants with Fresh Water Invertebrates (1); the OPPTS 850.1735: Whole Sediment Acute Toxicity Invertebrates, Freshwater (2); and Methods for Measuring the Toxicity and Bioaccumulation of Sediment-Associated Contaminants with Freshwater Invertebrates (2nd ed.) (3). Raw data for all work performed at Wildlife International, Ltd. and a copy of the final report will be filed by project number in archives located on the Wildlife International, Ltd. site, or at an alternative location to be specified in the final report.

OBJECTIVE

The objective of this study is to determine the effects of a sediment-incorporated test substance with a total organic carbon content of approximately 5%, on the amphipod, *Hyalella azteca*, during a 28 day exposure period under flow-through conditions. The measured endpoints of the test are survivorship and growth as determined by dry weight measurements.

EXPERIMENTAL DESIGN

Groups of amphipods will be exposed to a geometric series of at least five test concentrations, a negative (dilution water) control, and if necessary, a solvent control for approximately 28 days. Eight replicate test compartments will be maintained in each treatment and control group, with 10 amphipods in each compartment for a total of 80 individuals per test concentration. Each test compartment will contain a quantity of sediment and overlying water. Additional replicate test compartments may be included for analytical sampling of water and sediment. No amphipods will be placed in Day 0 replicates sampled at the beginning of the test. However, "analytical" replicates sampled at Day 7 and at the end of the test will contain amphipods. Test concentrations in the sediment will be prepared on a mg/kg dry weight basis.

Nominal test concentrations will be selected in consultation with the Sponsor, and will be based upon information such as the results of exploratory range-finding toxicity data, known toxicity data, physical/chemical properties of the test substance or other relevant information. Generally, each

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test substance concentration used in the definitive test will be at least 60% of the next higher concentration unless information concerning the concentration-effect curve indicates that a different dilution factor would be more appropriate. Overlying water, pore water, and sediment samples from the analytical sampling test compartments will be collected from the highest and lowest test concentrations. Day 0 samples may be collected slightly before organisms are added to the test system to accommodate analytical sampling schedules.

To control bias, amphipods will be impartially assigned to transfer chambers (e.g. 8 groups of 10 amphipods per chamber) which will then be assigned to exposure compartments at test initiation. No other potential sources of bias are expected to affect the results of the study. Survivorship and growth (mean individual dry weight) will be determined at the end of the test period (maximum of 28 days). LC50 values will be determined, when possible. The dose-response pattern and appropriate statistical analyses will be used to define the no-observed-effect-concentration (NOEC) and the lowest-observed-effect-concentration (LOEC). Nominal concentrations will be used to determine the LC50, NOEC and LOEC.

MATERIALS AND METHODS**Test Substance**

The test substance consisted of a composite of HBCD samples received from three manufacturers. The material's identity and date received from each of the manufacturers is given below:

<u>Manufacturer</u>	<u>Lot/Batch</u>	<u>Date Received</u>	<u>Wildlife International Ltd. Identification Number</u>
Great Lakes Chemical Corporation	Not Given	June 19, 1998	4515
Eurobrom b.v.	010328	December 10, 2001	5827
Albemarle Corporation	33449-15X	December 20, 1995	3519

The composite test substance was assigned Wildlife International Ltd. identification number 5850 and was stored under ambient conditions. A subsample of the composite test substance has been shipped to Albemarle Corporation for characterization and purity analyses. A copy of Albemarle Corporation's final report on characterization and purity determination will be included as an appendix to the results of these analyses.

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Preparation of Test Concentrations

Concentrations of the test substance in the sediment will be prepared on a dry weight basis (e.g., mg test chemical/Kg dry soil). Batch(es) of a suitable amount of dry sediment (ambient conditions) will be mixed with the test chemical for each test concentration. If necessary, the test substance will be dissolved in a volatile solvent (e.g. acetone) and mixed overnight so that the solvent can volatilize off the sediment during mixing. If an organic solvent is required, then a solvent control group will be included in the experimental design along with the negative (dilution water) control group. If used, the concentration of organic solvent will be minimized and will be the same in all treatment levels. The sediment and test substance will be mixed with a rotary mixer for an appropriate amount of time (e.g. overnight) for complete mixing to occur. Batches of treated sediment will be prepared for each treatment level and then transferred to test chambers. Approximately 100 ml of sediment will be placed in the bottom of each test compartment (300 ml beaker) and approximately 100- 150 ml of overlaying water will be slowly siphoned into the test compartment. After the test compartments are prepared, they will be transferred to a diluter unit and acclimated for approximately 48 hours before adding the test organisms. The length of the acclimation time may be shortened or lengthened based on the properties of the test substance.

Test Organism

The amphipod, *Hyaella azteca*, has been selected as the test species for this study. Amphipods represent an important group of aquatic invertebrates, and have been selected for use in the test based upon past use history and ease of culturing in the laboratory. Amphipods to be used in the test will be 7 to 14 days of age at test initiation. Amphipods will be fed 1.0 ml YCT (1.8 g/l) in a water suspension daily. The food ration will be reduced in all treatment levels and the control if fungal growth is seen in the control or any treatment group.

Specifications for acceptable levels of contaminants in YCT for amphipods have not been established. However, there are no levels of contaminants reasonably expected to be present in the diet that are considered to interfere with the purpose or conduct of the study.

Test Sediment

Artificial sediment, "Formulated Sediment A", as described in Kemble et al. (4) will be used as the test sediment. The soil will be composed of less than 1% humic acid and dolomite, and

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approximately 13% alpha-cellulose, 10% silt and clay (kaolin clay) and 77% industrial quartz sand. The dry constituents of the soil will be mixed in a PK Twinshell or equivalent mixer. Calcium carbonate will be added as needed to adjust the pH to 7.0 ± 0.5 . Total organic carbon content of the final mixture of sediment should be approximately 5%. Samples of the artificial sediment will be sent to a laboratory for analysis of total organic carbon.

Dilution Water

Water used for the holding and testing of amphipods will be obtained from a well approximately 40 meters deep located on the Wildlife International, Ltd. site. The water will be passed through a sand filter and pumped into a 37,800-L storage tank where the water will be aerated with spray nozzles. Prior to use the water will be filtered to $0.45 \mu\text{m}$ in order to remove fine particles. Water used for holding and testing is characterized as moderately hard. Typical values for hardness, alkalinity, pH and specific conductance are approximately:

Hardness, mg/L as CaCO_3	145
Alkalinity, mg/L as CaCO_3	190
pH	8.1
Specific Conductance, $\mu\text{mhos/cm}$	330

Hardness, alkalinity, pH and specific conductance will be measured weekly to monitor the consistency of the well water. Means and ranges of the measured parameters for the four-week period preceding the test will be provided in the final report. Analyses will be performed at least once annually to determine the concentrations of selected organic and inorganic constituents of the well water and results of the most recent GLP compliant analyses will be summarized in the final report.

Test Apparatus

The test apparatus will consist of a Wildlife International, Ltd. diluter unit that is designed to hold up to 14 tanks in a temperature controlled water bath. Each tank can receive renewal rates that can range from static to greater than 10 tank volume additions per day.

Test compartments will consist of 300 mL glass beakers with a nylon mesh covered hole on opposite sides of the beaker. Eight replicate beakers in each treatment group will be indiscriminately positioned in one or more diluter tanks. Beakers will be labeled with the project number, test

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concentration and replicate. Each beaker will contain approximately 100 ml of sediment and approximately 100 to 150 ml of overlying water. The water level in the beakers will be maintained by the water levels in diluter tanks. Volume additions in diluter tanks will be adjusted to result in approximately two or less volume additions per day. A glass siphon cap will be positioned in each diluter tank to create passive flow of dilution water through the holes in the sides of the beakers and exchange of the water overlying the sediment. Loose plastic covers will be placed over each test compartment during the test.

Environmental Conditions

Lighting used to illuminate the cultures and test chambers in test compartments during culturing and testing will be provided by fluorescent tubes that emit wavelengths similar to natural sunlight (e.g., Colortone® 50). A photoperiod of 16 hours of light and 8 hours of dark will be controlled with an automatic timer. A 30-minute transition period of low light intensity will be provided when lights go on and off to avoid sudden changes in light intensity. Light intensity will be measured at test initiation with a SPER Scientific Ltd. light meter or equivalent and should fall within the range of 100 to 1000 lux.

The target test temperature will be $23 \pm 2^{\circ}\text{C}$. Temperature will be measured using a mercury or alcohol thermometer in the overlying water in one replicate test chamber of each experimental group at the beginning and end of the test and at least three times per week during the test. Temperature also will be measured with a continuous recorder in a beaker of water placed adjacent to the test chambers. Recorder measurements will be verified with a hand-held thermometer prior to test initiation.

Dissolved oxygen will be measured in one replicate of each experimental group at the beginning and end of the test and at least three times per week during the test using a Yellow Springs Instrument Model 51B dissolved oxygen meter, or equivalent. In the event that dissolved oxygen levels fall below 60% saturation, dissolved oxygen measurements will be made in every test chamber. An attempt will be made to notify the Sponsor by telephone call or facsimile of the condition of the test and the measured dissolved oxygen concentrations. A judgment will be made as to the severity of the condition and its impact on the test and whether aeration is necessary. The outcome of that judgment will be documented in the study records.

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Measurements of pH, ammonia, hardness, alkalinity and specific conductance will be measured at the beginning and end of the test in a sample of the overlying water from one replicate in each experimental group. Measurements of pH also will be measured at least once per week in a sample of the overlying water from one replicate in each experimental group. A Fisher Accumet Model 915 pH meter, or equivalent will be used to measure pH. Ammonia will be measured with a ThermoOrion Model 720A Ion Selective Meter and ammonia probe, or equivalent. Hardness and alkalinity measurements will be made by titration using procedures based on methods in *Standard Methods for the Examination of Water and Wastewater* (5). Specific conductance will be measured using a Yellow-Springs Instrument Model 33 Salinity-Conductivity-Temperature meter, or equivalent.

If a treatment group reaches 100% mortality, then the dissolved oxygen, pH and temperature measurements will be collected at that time and then measurements will be discontinued for the remainder of the test.

Test Procedures and Biological Measurements

After the settling period for the water/sediment systems, one to two 7 to 14 day old amphipods will be sequentially added to a glass beaker until each beaker contains its complement of 10 individuals. The individuals then will be transferred below the air/water interface to the test compartments. Test compartments will be observed at least three times per week to make visual assessments of any abnormal behavior (e.g. leaving sediment, unusual swimming).

Observations will be made at test termination to determine the number of mortalities and the number of individuals exhibiting clinical signs of toxicity or abnormal behavior. On Day 28, amphipods will be segregated from the sediment and live and dead organisms will be enumerated. When the total number of individuals found in each replicate at test termination are fewer than the number initially placed in each replicate, then those missing will be considered dead. Dry weights will be determined for surviving amphipods in each replicate to evaluate effects on growth.

Sampling for Analytical Measurements

If stock solutions are used to spike sediment, samples of the stock solutions will be collected and analyzed on the day the sediments are dosed. Overlying water, pore water, and sediment samples

Wildlife International, Ltd.

from the analytical sampling test compartments will be collected from the highest and lowest test concentrations. Day 0 samples may be collected slightly before organisms are added to the test system to accommodate analytical schedules. The overlying water will be sampled, then the remaining sediment will be collected, centrifuged, and split into separate samples of pore water and sediment. If samples are not analyzed immediately, then samples will be placed in an appropriate container and stored frozen until analyzed. Stock solution samples will be analyzed as soon as possible after collection. The sampling scheme is summarized below:

PROPOSED NUMBERS OF VERIFICATION SAMPLES									
Experimental Group	Day 0			Day 7			Termination (28)		
	Wat	Sed	Pore	Wat	Sed	Pore	Wat	Sed	Pore
Stocks	5	-	-	-	-	-	-	-	-
Control	1	2	2	1	2	2	1	2	2
Solvent Control (if needed)	1	2	2	1	2	2	1	2	2
Level 1-Low Concentration	1	2	2	1	2	2	1	2	2
Level 2	-	-	-	-	-	-	-	-	-
Level 3	-	-	-	-	-	-	-	-	-
Level 4	-	-	-	-	-	-	-	-	-
Level 5-High Concentration	1	2	2	1	2	2	1	2	2
	9	8	8	4	8	8	4	8	8

The above numbers of samples represent those collected from the test and do not include quality control (QC) samples such as matrix blanks and fortifications prepared and analyzed during the analytical chemistry phase of the study.

Analytical Method Development and Verification

Wildlife International, Ltd. will develop appropriate analytical methods and validate them for Sponsor approval prior to their use in support of this study. If the Sponsor provides an analytical method, Wildlife International, Ltd. will demonstrate its validity to the Sponsor before being used in support of this study. All analytical methods accepted for use in this study will be added by protocol amendment and described in detail as an Appendix to the final report.

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Analytical Chemistry

Chemical analysis of the samples will be performed by Wildlife International, Ltd. The analytical method used will be based upon chromatographic methodology provided by the Sponsor and/or developed at Wildlife International, Ltd. The methodology used to analyze the test samples will be documented in the raw data and summarized in the final report.

Data Analysis

When the dose-response pattern allows calculation of an EC50 value, the data will be analyzed using the computer software of C.E. Stephan (6). The program was designed to calculate the EC50 value and the 95% confidence interval by probit analysis, the moving average method, or binomial probability with nonlinear interpolation (7,8,9). The EC50 value will be calculated, when possible, using mortality data collected at the end of the test.

Analyses of mortality and growth (mean individual dry weight) data will be evaluated for normality and homogeneity of variances. If the data are deemed normal with homogeneous variances, hypothesis testing using analysis of variance (ANOVA) and multiple means tests (e.g., Dunnett's, Bonferroni, Scheffe) will be used. If the data fail the tests for normality or homogeneity, then data transformations will be tried in an attempt to correct the condition. When the data transformations fail to correct for non-normality or heterogeneity of variances, nonparametric procedures will be used to identify statistically significant differences among the experimental groups.

RECORDS TO BE MAINTAINED

Records to be maintained for data generated by Wildlife International, Ltd. will include, but not be limited to:

1. A copy of the signed protocol.
2. Identification and characterization of the test substance, if provided by the Sponsor.
3. Dates of initiation and termination of the test.
4. *Hyalella azteca* culture records.
5. Stock solution calculation and preparation, if applicable.
6. Biological observations.
7. Water chemistry results (e.g., hardness and alkalinity).

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8. Statistical calculations, if applicable.
9. Test conditions (light intensity, photoperiod, etc.).
10. Calculation and preparation of test concentrations.
11. Copy of final report.

FINAL REPORT

A final report of the results of the study will be prepared by Wildlife International, Ltd. The report will include, but not be limited to, the following, when applicable:

1. Name and address of the facility performing the study.
2. Dates upon which the study was initiated and completed, and the definitive experimental start and termination dates.
3. A statement of compliance signed by the Study Director addressing any exceptions to Good Laboratory Practice Standards.
4. Objectives and procedures, as stated in the approved protocol, including all changes to the protocol.
5. The test substance identification including name, chemical abstract number or code number, strength, purity, composition, and other information provided by the Sponsor.
6. A copy of the protocol and protocol amendments.
7. Stability and solubility of the test substance under the conditions of administration, if provided by the Sponsor.
8. A description of the methods used to conduct the test.
9. A description of the test organisms, including the source, scientific name, age or life stage, feed types, light intensity and photoperiod.
10. A description of the preparation of the test solutions.
11. The methods used to allocate organisms to test chambers and begin the test, the number of organisms and compartments per chamber per treatment, and the duration of the test.
12. A description of circumstances that may have affected the quality or integrity of the data.
13. The name of the Study Director and the names of other scientists, professionals, and supervisory personnel involved in the study.
14. A description of the transformations, calculations, and operations performed on the data, a summary and analysis of the biological data and analytical chemistry data, and a statement of the conclusions drawn from the analyses.

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15. Statistical methods used to evaluate the data.
16. The signed and dated reports of each of the individual scientists or other professionals involved in the study, if applicable.
17. The location where raw data and final report are to be stored.

CHANGES TO PROTOCOL

Planned changes to the protocol will be in the form of written amendments signed by the Study Director and approved by the Sponsor's Representative. Amendments will be considered as part of the protocol and will be attached to the final protocol. Any other changes will be in the form of written deviations signed by the Study Director and filed with the raw data. All changes to the protocol will be indicated in the final report.

GOOD LABORATORY PRACTICES

This study will be conducted in accordance with Good Laboratory Practice Standards for EPA (40 CFR Part 160 and/or Part 792); OECD Principles of Good Laboratory Practice (ENV/MC/CHEM(98) 17); and Japan MAFF (59 NohSan, Notification No. 3850, Agricultural Production Bureau). Each study conducted by Wildlife International, Ltd. is routinely examined by the Wildlife International, Ltd. Quality Assurance Unit for compliance with Good Laboratory Practices, Standard Operating Procedures and the specified protocol. A statement of compliance with Good Laboratory Practices will be prepared for all portions of the study conducted by Wildlife International, Ltd. The Sponsor will be responsible for compliance with Good Laboratory Practices for procedures performed by other laboratories (e.g., residue analyses or pathology). Raw data for all work performed at Wildlife International, Ltd. and a copy of the final report will be filed by project number in archives located on the Wildlife International, Ltd. site, or at an alternative location to be specified in the final report.

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REFERENCES

- 1 ASTM E 1706-95b. 1995. *Standard Test Methods for Measuring the Toxicity of Sediment-Associated Contaminants with Fresh Water Invertebrates*. in 1997 Annual Book of ASTM Standards, Section 11 Water and Environmental Technology, Volume 11.05 Biological Effects and Environmental Fate; Biotechnology; Pesticides.
- 2 U.S. Environmental Protection Agency. 1996. Series 850 - Ecological Effects Test Guidelines (*draft*), OPPTS Number 850.1735: Whole Sediment Acute Toxicity Invertebrates, Freshwater.
- 3 U.S. Environmental Protection Agency. 2000. *Methods for Measuring the Toxicity and Bioaccumulation of Sediment-associated Contaminants with Freshwater Invertebrates*. (2nd ed.) Office of Research and Development Mid-Continent Ecology Division, Duluth, Minnesota and Office of Science and Technology Office of Water, Washington D.C. EPA 600-99/064.
- 4 Kemble, N.E., F.J. Dwyer, C.G. Ingersoll, T.D. Dawson, and T.J. Norberg-King. 1999. *Tolerance of Freshwater Test Organisms to Formulated Sediments for use as Control Materials in Whole-Sediment Toxicity Tests*. Environ. Toxicol. Chem. 18:222-230.
- 5 APHA, AWWA, WPCF. 1985. *Standard Methods for the Examination of Water and Wastewater*. 16th Edition, American Public Health Association. American Water Works Association. Water Pollution Control Federation, New York.
- 6 Stephan, C.E. 1978. U.S. EPA, Environmental Research Laboratory, Duluth, Minnesota. Personal communication.
- 7 Thompson, W.R. 1947. *Bacteriological Reviews*. Vol. II, No. 2. Pp. 115-145.
- 8 Stephan, C.E. 1977. "Methods for Calculating an LC50," *Aquatic Toxicology and Hazard Evaluations*. American Society for Testing and Materials. Publication Number STP 634, pp 65-84.
- 9 Finney, D.J. 1971. *Statistical Methods in Biological Assay*. Second edition. Griffin Press, London.

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APPENDIX I

IDENTIFICATION OF TEST SUBSTANCE BY SPONSOR

To be Completed by Sponsor

- I. Test Substance Identity (name to be used in the report): HBCD
Test Substance Sample Code or Batch Number: Wildlife International Ltd. Identification No. 5850
Test Substance Purity (% Active Ingredient): to be determined Expiration Date: _____
- II. Test Substance Characterization
Have the identity, strength, purity and composition or other characteristics which appropriately define the test substance and reference standard been determined prior to its use in this study in accordance with GLP Standards? ☒ Yes ☐ No
- III. Test Substance Storage Conditions
Please indicate the recommended storage conditions at Wildlife International, Ltd.
Ambient
Has the stability of the test substance under these storage conditions been determined in accordance with GLP Standards? ☐ Yes ☐ No
Other pertinent stability information: _____
- IV. Test Concentrations: _____ Adjust test concentration to 100% a.i. based upon the purity (%) given above.
_____ ☒ Do not adjust test concentration to 100% a.i. Test the material AS IS.
- V. Toxicity Information:
Mammalian: Rat LD50 _____ Mouse LD50: _____
Aquatic: Invertebrate Toxicity (EC/LC50) _____ Fish Toxicity (LC50) _____

Other Toxicity Information (including findings of chronic and subchronic tests): _____
- VI. Classification of the Compound:
_____ Insecticide _____ Herbicide _____ Fungicide
_____ Microbial Agent _____ Economic Poison
Other: Flame retardant

Wildlife International, Ltd.

Project Number 439A-120
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AMENDMENT TO STUDY PROTOCOL

STUDY TITLE: HEXABROMOCYCLODODECANE (HBCD): A Prolonged Sediment Toxicity Test with *Hyaella azteca* Using Spiked Sediment with 5% Total Organic Carbon

PROTOCOL NO.: 439/010302/HYA-SED5%/SUB439

AMENDMENT NO.: 1

SPONSOR: American Chemistry Council's
Brominated Flame Retardant Industry Panel


PROJECT NO.: 439A-120

EFFECTIVE DATE: January 17, 2003

AMENDMENT: Page 2:

ADD: Proposed Dates: Experimental Start Date: January 15, 2003
Experimental Termination Date: February 12, 2003
Test Concentrations: 31, 63, 125, 250, 500, 1000 mg of HBCD/Kg
Test Substance No.: 5850
Reference Substance No.: 5827

REASON: This information was not available at the time the protocol was signed by the Study Director.


STUDY DIRECTOR

17 Jan 03
DATE


LABORATORY MANAGEMENT

1/17/03
DATE

Wildlife International, Ltd.

Project No.: 439A-120
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DEVIATION TO STUDY PROTOCOL

STUDY TITLE: Hexabromocyclododecane (HBCD): A Prolonged Sediment Toxicity Test With *Hyalella azteca* Using Spiked Sediment With 5% Total Organic Carbon

PROTOCOL NO.: 439/010302/HYA-SED5%/SUB439

DEVIATION NO.: 1

SPONSOR: American Chemistry Council's Brominated Flame Retardant Industry Panel

PROJECT NO.: 439A-120

DATE OF DEVIATION: January 22, 2003

DEVIATION: A transfer beaker was not used to transfer organisms from the culture to test chambers. Instead, organisms were directly transferred from the culture to the test chambers.

REASON: Direct transfer was performed since it reduces stress to the organisms. This deviation from the protocol had no adverse impact upon the results or interpretation of the study.

DEVIATION: The protocol stated that the volume additions in the diluter tanks would result in two or less volume additions per day and did not clearly indicate that the volume additions pertained to test chambers within the tank. Two or less volume additions were maintained in test chambers throughout the test.

REASON: Protocol clarification. This deviation from the protocol had no adverse impact upon the results or interpretation of the study.

DEVIATION: Continuous temperature measurements were made directly in the negative control diluter tank instead of a beaker beside the test chambers.

REASON: Biologist oversight. This deviation from the protocol had no adverse impact upon the results or interpretation of the study.

DEVIATION: Overlying water samples were analyzed in duplicate along with samples of pore water and sediment. The proposed schedule for sample analysis listed only one overlying water sample, instead of two.

REASON: The additional samples were analyzed to show the consistency of results. This deviation from the protocol had no adverse impact upon the results or interpretation of the study.

Wildlife International, Ltd.

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DEVIATION: A 1.0 mL aliquot of food was added to each test compartment daily throughout the test except on days 6, 12, 14 and 16. On day 6, the food was reduced to 0.5 mL due to a lack of availability of food. On days 12, 14 and 16, the food was withheld due to fungal growth.


REASON: The protocol indicated that 1.0 mL would be given daily, and that if fungal growth were observed the food ration would be reduced. The above changes in feeding regimes had no adverse impact upon the results or interpretation of the study.

DEVIATION: Analytical method development and approval of the method by the Sponsor as stated in the protocol were not done prior to use. Additionally, the accepted methods were not amended to the protocol.

REASON: Analytical methods had been developed for soil and water on previous studies, therefore it was not necessary for the methods to be validated and approved by the Sponsor for this study. The analytical methods were not amended to the study protocol, but are fully described in the final report. This deviation from the protocol had no adverse impact upon the results or interpretation of the study.

DEVIATION: Additional statistical analyses (Fisher's Exact Test) were used in the analysis of survival/mortality data.

REASON: The additional procedure was more appropriate. This deviation from the protocol had no adverse impact upon the results or interpretation of the study.



STUDY DIRECTOR

5/2/03

DATE



LABORATORY MANAGEMENT

5/2/03

DATE

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APPENDIX 2

Test Substance Characterization

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CERTIFIED TRUE COPY

pfl 2/21/02

ALBEMARLE CORPORATION
RESEARCH AND DEVELOPMENT DEPARTMENT

FINAL REPORT ON THE CHEMICAL CHARACTERIZATION (IDENTITY AND
HOMOGENEITY) OF HEXABROMOCYCLODODECANE (HBCD), WIL TEST
SUBSTANCE #5850, COMPOSITED FROM WIL #3519, 4515 AND 5827

- I. Reference Protocol Number: HBCD-01-15-2002
- II. Sponsor: American Chemistry Council
Brominated Flame Retardant Industry Panel
1300 Wilson Boulevard
Arlington, Virginia 22209
Study Monitor: Wendy K. Sherman
- III. Analytical Testing Facilities: Albemarle Corporation
Albemarle Technical Center
8000 GSRI Avenue
Baton Rouge, LA 70820
Study Chemist: Paul F. Ranken, Ph. D.
- IV. Dates of Performance: Study Initiation Date: January 30, 2002
Study Completion Date: February 21, 2002
- V. Test Article: Hexabromocyclododecane (WIL Test Substance #5850). The test article is a composite of WIL #3519, 4515, and 5827 which are samples of commercial products from Albemarle Corporation, Great Lakes Chemical Corporation and Eurobrom B.V. The composite was prepared by Wildlife International Ltd., Easton, MD 21601.
- VI. Objective/Methodology: This study was initiated to confirm the identity of the test article and to demonstrate the homogeneity of the test article. The identity of one sample of the test article, taken from the

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middle center of the bulk container and designated "Characterization Sample #MC158500102", was confirmed by Fourier Transform Infrared Spectroscopy using SOP No. ARS-284-R4. In this procedure, the sample infrared spectrum was compared to a standard reference spectrum of HBCD. The HBCD infrared spectrum published in the **Aldrich Library of FT-IR Spectra**, Volume 1, page 107A, was used as the reference spectrum. The homogeneity of the test article was demonstrated by determining the composition (area % of the three HBCD diastereomers) of six separate test article samples which were taken from the top, middle and bottom right side and from the top, middle and bottom left side of the bulk container. The composition of the six samples was determined by High Performance Liquid Chromatography (HPLC) using SOP No. ARS-432-R1. Each of the six samples was analyzed in triplicate and the composition of each sample expressed as the average area % of each of the three diastereomers. Chain of Custody and sample handling were conducted according to established standard operating procedures.

VII. Results:

The attached Conclusions and test article analytical data contain all of the test results from the study. The identity of the test article was confirmed by Fourier Transform Infrared Spectroscopy. The homogeneity of the test article was confirmed by HPLC analysis; all six test article samples had the same composition (<5 % difference of the HBCD diastereomers area % for each sample compared to the average HBCD diastereomer area % of the six samples). There were no circumstances that may have affected the quality or integrity of the data.

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VIII. Deviations:

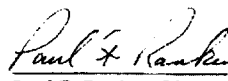
Two deviations occurred from SOP No. ARS-432-R1. The SOP required that each sample be analyzed in duplicate. Each sample was analyzed in triplicate, as specified in the analytical protocol. In addition, the SOP required that a representative sample be labeled as a "lab/reference standard" and be analyzed. Pass or failure of subsequent samples in the study was to be determined by comparison to this original sample. The representative sample was not designated nor analyzed and no comparisons to subsequent samples were made. The pass/fail criterion for the test article in this study was outlined in the analytical protocol and was not based on a "lab/reference standard". The pass/fail criterion in SOP No. ARS-432-R1 was not applicable to this study. These deviations did not affect the quality or integrity of the data generated.

IX. Regulatory Requirements:

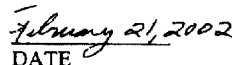
The study conformed to the requirements of EPA TSCA (40 CFR Part 792) Good Laboratory Practice Regulations and the OECD [C(97)186/Final] Good Laboratory Practice Regulations.

X. Data/Record Retention:

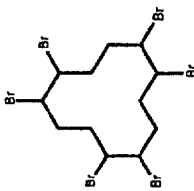
All original log books, spectra and reports will be forwarded to the Quality Assurance Unit (QAU) for a final review prior to filing in the designated Health and Environment archives at Albemarle Corporation, Health and Environment Department, 451 Florida Street, Baton Rouge, LA 70801.



Paul F. Ranken, Ph. D.
STUDY CHEMIST


DATE

CONCLUSIONS AND TEST ARTICLE ANALYTICAL DATA

CHEMICAL NAME: Hexabromocyclododecane TEST ARTICLE: HBCD Composite, WIL Test Substance #5850 C.A.S. No.: 3194-55-6 MOLECULAR FORMULA: $C_{12}H_{18}Br_6$ PHYSICAL FORM: White Powder CHEMICAL STRUCTURE: <div style="text-align: center;">  </div>						
ANALYSIS	RESULTS				ANALYSIS DATES	ANALYST
FT-IR	The sample FT-IR spectrum matched that of the Aldrich reference spectrum. All spectra are on file with the original data.				02/13/02	W. T. Cobb
HPLC	Area %				02/14/02	J. S. Arroyave
	Alpha Average	% Difference from Mean	Beta Average	% Difference from Mean	Gamma Average	% Difference from Mean
Top Right	5.83	0.69	19.33	0.05	74.83	0.08
Middle Right	5.67	2.07	19.51	0.98	74.82	0.09
Bottom Right	5.84	0.86	19.61	1.5	74.55	0.45
Top Left	5.91	2.07	19.57	1.29	74.52	0.49
Middle Left	5.85	1.04	19.44	0.62	74.71	0.24
Bottom Left	5.64	2.59	18.42	4.66	75.94	1.4
Mean	5.79		19.32		74.89	
CONCLUSION: Based on these analytical data, the test article was identified as HBCD. The test article was homogeneous.						

Test Article HPLC Analytical Data

Sample	Alpha Area %	Beta Area %	Gamma Area %
Top Right	5.86	19.65	74.48
Top Right	5.8	18.95	75.25
Top Right	5.84	19.4	74.75
Average	5.83	19.33	74.83
Middle Right	5.52	19.44	75.04
Middle Right	5.78	19.53	74.69
Middle Right	5.7	19.57	74.73
Average	5.67	19.51	74.82
Bottom Right	5.98	19.6	74.42
Bottom Right	5.99	19.71	74.3
Bottom Right	5.54	19.53	74.93
Average	5.84	19.61	74.55
Top Left	5.95	19.33	74.72
Top Left	5.91	20.44	73.65
Top Left	5.87	18.93	75.2
Average	5.91	19.57	74.52
Middle Left	5.92	19.08	75
Middle Left	5.8	20.37	73.83
Middle Left	5.83	18.87	75.29
Average	5.85	19.44	74.71
Bottom Left	5.73	17.56	76.71
Bottom Left	5.66	19.99	74.36
Bottom Left	5.53	17.72	76.74
Average	5.64	18.42	75.94
Mean (n = 18)	5.79	19.32	74.89

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APPENDIX 3

Artificial Soil Preparation

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APPENDIX 3**Artificial Soil Preparation¹**

Constituents	Weight (g)
Quartz Sand	15,400
Kaolin Clay	2,000
Alpha Cellulose	2,600
Dolomite	100
Humic Acid	2.0

¹ The constituents were mixed in a PK Twinshell mixer 20 minutes and the dry soil was stored under ambient conditions until used.

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APPENDIX 4

Specific Conductance, Hardness, Alkalinity and pH of Well Water Measured
During the 4-Week Period Immediately Preceding the Test

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APPENDIX 4

Specific Conductance, Hardness, Alkalinity and pH of Well Water Measured
During the 4-Week Period Immediately Preceding the Test

	Mean	Range
Specific Conductance (μ mhos/cm)	313 (N = 4)	310 – 315
Hardness (mg/L as CaCO ₃)	133 (N = 4)	132 – 136
Alkalinity (mg/L as CaCO ₃)	173 (N = 4)	170 – 178
pH	8.3 (N = 4)	8.1 – 8.4

APPENDIX 5

Analyses of Pesticides, Organics and Metals
in Wildlife International, Ltd. Well Water

APPENDIX 5

Analyses of Pesticides, Organics and Metals in Wildlife International, Ltd. Well Water¹

Pesticides and Organics			
Component	Measured Concentration (ppb or ng/g)	Component	Measured Concentration (ppb or ng/g)
Aldicarb sulfone	< 50	Isofenphos	< 50
Aldicarb sulfoxide	< 50	Leptophos	< 50
Azinphos-ethyl	< 50	Linuron	< 50
Azinphos-methyl	< 50	Methidathion	< 50
Bifenox	< 50	Methiocarb	< 50
Bitertanol	< 50	Methomyl	< 50
Bromacil	< 50	Methoxychlor	< 250
Bromoxynil octanoic acid ester	< 50	Mirex	< 50
Captafol	< 50	Monocrotophos	< 50
Carbaryl	< 50	Myclobutanil	< 50
3-Hydroxy Carbofuran	< 50	Napropamide	< 50
Carbofuran	< 50	Norflurazon	< 50
Carbophenothion	< 50	Oxadiazon	< 50
cis-Chlordane	< 50	Oxamyl	< 50
trans-Chlordane	< 50	Oxyfluorfen	< 50
Chlorfenson	< 50	Paraoxon	< 50
trans-Chlorfenvinphos	< 50	cis-Permethrin	< 50
Chlorobenzilate	< 50	Perthane	< 50
Chloropropylate	< 50	Phosalone	< 50
Chloroxuron	< 50	Phosphamidon	< 50
Coumaphos	< 50	Piperalin	< 50
Crotoxyphos	< 50	Profenfos	< 50
Cyanazine	< 50	Promecarb	< 50
Cyfluthrin I	< 50	Propanil	< 50
Cypermethrin I	< 50	Propargite	< 50
o,p'-DDD	< 50	Propoxur	< 50
p,p'-DDE	< 50	Pyrethrin I	< 50
p,p'-DDD	< 50	Quinalphos	< 50
o,p'-DDT	< 50	Quinomethionate	< 50
p,p'-DDT	< 250	Quizalofop-ethyl	< 50
DEF	< 50	Sulprofos	< 50
Diclofop methyl	< 50	Tetrachlorovinphos	< 50
Dicrotophos	< 50	Tetradifon	< 50
Dieldrin	< 50	Thiobendazole	< 50
Diphenamid	< 50	Tilt I	< 50
Diuron	< 50	Tilt II	< 50
Endosulfan II	< 50	Trimethyl carbamate	< 50
Endrin	< 50		
Endrin ketone	< 50		
EPN	< 50		
Ethion	< 50		
Fenamiphos	< 50		
Fenarimol	< 50		
Fenobucarb	< 50		
Fenpropathrin	< 50		
Fensulfothion	< 50		
Fluzifop-P-butyl	< 50		

¹Analyses performed by Exygen Research on samples collected on July 31, 2002.

APPENDIX 5 (continued)Analyses of Pesticides, Organics and Metals in Wildlife International, Ltd. Well Water¹

Metals			
	(ppm or mg/L)		(ppm or mg/L)
Aluminum	< 0.204	Manganese	< 0.0153
Arsenic	< 0.0102	Mercury	< 0.0002
Beryllium	< 0.0051	Molybdenum	< 0.0005
Cadmium	< 0.0051	Nickel	< 5.1
Calcium	28.2	Potassium	5.45
Chromium	< 0.0102	Selenium	0.009
Cobalt	< 5.1	Silver	< 0.0102
Copper	< 0.0255	Sodium	18.6
Iron	< 5.1	Zinc	< 0.0204
Magnesium	11.6		

¹Analyses performed by Exygen Research on samples collected on July 31, 2002.

APPENDIX 6

The Analysis of Hexabromocyclododecane in Water and Sediment

APPENDIX 6.1**Typical HPLC Operational Parameters for the Analysis of Hexabromocyclododecane (HBCD) in
Freshwater and Sediment**

INSTRUMENT:	Agilent Model 1100 High Performance Liquid Chromatograph (HPLC) equipped with an Agilent Series 1100 Variable Wavelength Detector			
ANALYTICAL COLUMN:	YMC Pack ODS-AM Column (150 mm x 4.6 mm, 3 µm particle size)			
STOP TIME:	15 minutes			
FLOW RATE:	1.000 mL/min			
OVEN TEMPERATURE:	40°C			
SOLVENT A:	0.1% H ₃ PO ₄			
SOLVENT B:	CH ₃ CN			
GRADIENT:	Time (min.)	% A	% B	Flow (mL/min.)
	0.01	50.0	50.0	1.000
	1.00	50.0	50.0	1.000
	6.00	2.0	98.0	1.000
	11.00	2.0	98.0	1.000
	11.10	50.0	50.0	1.000
	15.00	50.0	50.0	1.000
INJECTION VOLUME:	100 µL			
APPROXIMATE HBCD RETENTION TIMES:	Sediment: 10.3 minutes Water: 10.7 minutes			
DETECTION CONDITIONS:	Wavelength - 220 nm			

APPENDIX 6.2

**Analytical Method Flowchart for the Analysis of Hexabromocyclododecane
(HBCD) in Sediment**

**METHOD OUTLINE FOR THE ANALYSIS OF
HEXABROMOCYCLODODECANE (HBCD) IN SEDIMENT**

Prepare all glassware by successively rinsing with a 5% solution of KOH in methanol (base), followed by tap water, followed by 10% HCL (acid), followed by tap water, followed by acetone, followed by THF. Allow glassware to set or remove solvent residues using nitrogen.



Prepare samples and QC's by weighing 2.0 g of soil into scintillation vials. Fortify QC's using the appropriate stock solution. Unfortified soil will serve as the matrix blank.



Add 10.0 mL of THF and sonic disrupt each sample for approximately 10 minutes.



Centrifuge each sample for ~ 5 minutes at ~1500 rpm. Transfer extract to a roundbottom flask.



Repeat the above extraction with an additional 10 mL of THF. Add centrifuged extract to appropriate roundbottom flask.



Rotary evaporate each sample to approximately 0.50 – 1.0 mL using a waterbath maintained at approximately 40°C. Do not rotary evaporate to dryness.



Evaporate the samples to dryness under a gentle stream of nitrogen.



Volumetrically add the requisite volume of THF to each roundbottom flask and swirl well in order to dissolve all residues. Filter samples (if necessary) at this point. Perform secondary dilutions in 50% THF : 50% water.



Transfer an aliquot of each extract to an autosampler vial. Submit samples of HPLC/UV analysis.

APPENDIX 6.3**Analytical Stock and Standards Preparation**

Stock solutions of HBCD were prepared by weighing 0.1002 g of the test substance on an analytical balance. The test substance was transferred to a 100-mL class A volumetric flask and brought to volume using THF. The primary stock solutions contained 1.00 mg/mL of HBCD. These primary stock solutions were diluted with THF to prepare secondary stock solutions at a concentration of 0.100 mg/mL. Matrix fortification samples and calibration standards were prepared in 50% THF: 50% H₂O. The following shows the dilution scheme for each set of calibration standards:

Stock Concentration (mg/mL)	Aliquot (μ L)	Final Volume (mL)	Standard Concentration (mg/L)
0.100	100	10.0	1.00
0.100	250	10.0	2.50
0.100	500	10.0	5.00
0.100	750	10.0	7.50
0.100	1000	10.0	10.0

APPENDIX 6.4**Example Calculations for a Representative Sediment Sample**

The analytical result and percent recovery for sample number 149A-138-S-4, with a nominal concentration of 31 µg/g, were calculated using the following equations:

$$\text{HBCD in sample (}\mu\text{g/g)} = \frac{\text{Peak Area} - \text{Y-intercept}}{\text{Slope}} \times \text{Dilution Factor}$$

$$\text{Percent of nominal concentration} = \frac{\text{measured concentration of HBCD in sample (}\mu\text{g/g)}}{\text{nominal concentration of HBCD in sample (}\mu\text{g/g)}} \times 100$$

Peak Area = 14.79940

Y-intercept = -0.1538

Slope = 7.62

Dilution factor = 12.5

Soil = Dry Soil Mass (g)/Wet Soil Mass (g) = 11.88 g/7.451 g = 0.627

$$\text{HBCD in sample (}\mu\text{g/g)} = \frac{14.79940 + 0.1538}{7.62} \times \frac{12.5}{0.627}$$

$$\text{Concentration of HBCD in sample (}\mu\text{g/g)} = 39.1 \mu\text{g/g}$$

$$\text{Percent of nominal concentration} = \frac{39.1 \mu\text{g/g}}{31 \mu\text{g/g}} \times 100$$

$$\text{Percent of nominal concentration} = 126\%$$

APPENDIX 6.5

Matrix Blanks and Fortifications Analyzed Concurrently During Sample Analyses in Sediment

Sample Number (439A-120-)	Sampling Interval (Day)	Concentrations of HBCD		Percent Recovery ²
		Fortified (µg/g)	Measured ¹ (µg/g)	
SMAB-1	0	0	< LOQ	--
SMAB-2	7	0	< LOQ	--
SMAB-3	28	0	< LOQ	--
SMAS-1	0	30.0	30.7	102
SMAS-2	0	1000	998	99.8
SMAS-3	7	30.0	30.0	100
SMAS-4	7	1000	988	98.8
SMAS-5	28	30.0	29.6	98.7
SMAS-6	28	1000	985	98.5
Mean =				99.6
Standard Deviation =				1.31
CV =				1.32

¹ The limit of quantitation (LOQ) was 12.5 µg/g, calculated as the product of the concentration of the lowest calibration standard (1.00 mg/L) and the product of the dilution factor of the matrix blank samples (12.5).

² Results were generated using Excel 2000 in the full precision mode. Manual calculations may differ slightly.

APPENDIX 6.6

Matrix Blanks and Fortifications Analyzed Concurrently During Sample Analyses in Freshwater

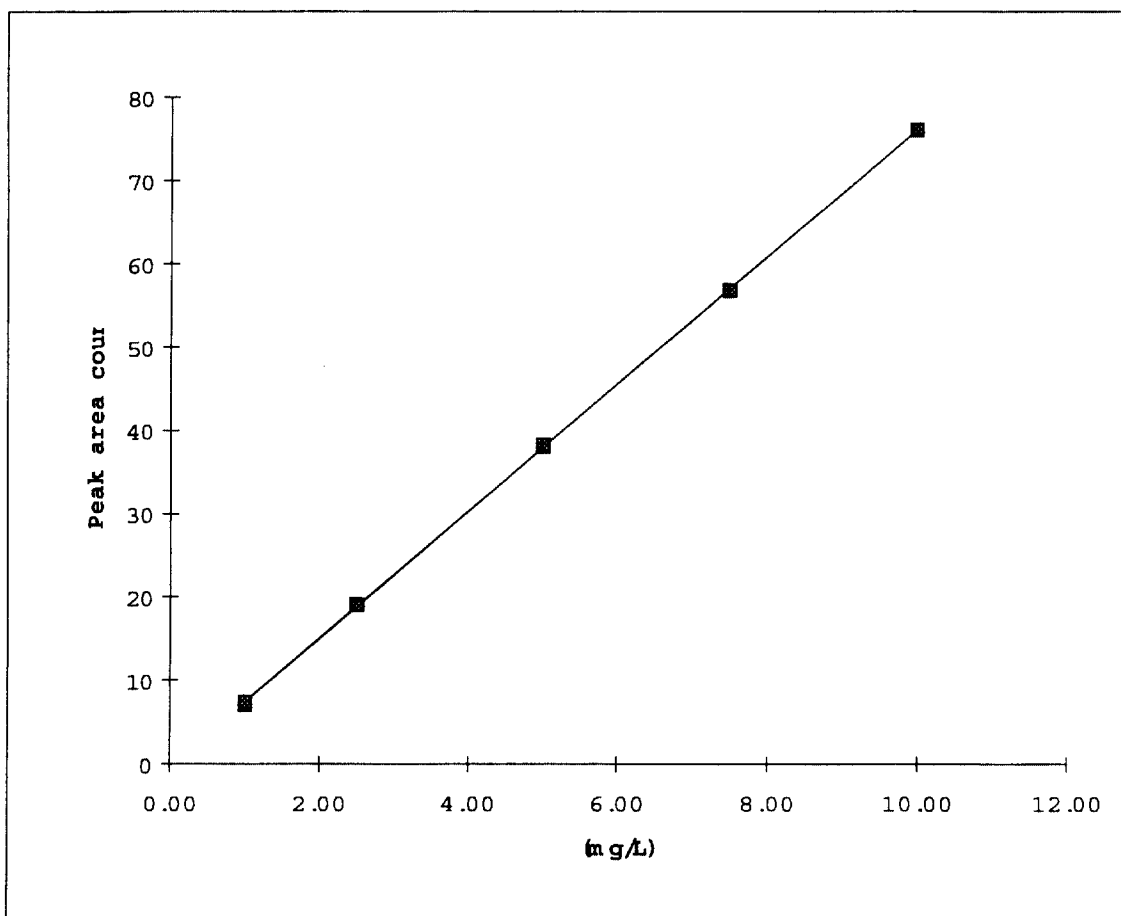
Sample Number (439A-120-)	Sampling Interval (Day)	Concentrations of HBCD		Percent Recovery ²
		Fortified (mg/L)	Measured ¹ (mg/L)	
MAB-1	0	0	< LOQ	--
MAB-2	7	0	< LOQ	--
MAB-3	28	0	< LOQ	--
MAS-1	0	0.200	0.162	81.2
MAS-2	0	0.500	0.334	66.7
MAS-3	7	0.200	0.198	98.8
MAS-4	7	0.500	0.482	96.3
MAS-5	28	0.200	0.204	102
MAS-6	28	0.500	0.515	103
Mean =				91.3
Standard Deviation =				14.4
CV =				15.8

¹ The limit of quantitation (LOQ) was 0.100 mg/L, calculated as the product of the concentration of the lowest calibration standard (1.00 mg/L) and the dilution factor of the matrix blank samples (0.100).

² Results were generated using Excel 2000 in the full precision mode. Manual calculations may differ slightly.

APPENDIX 6.7

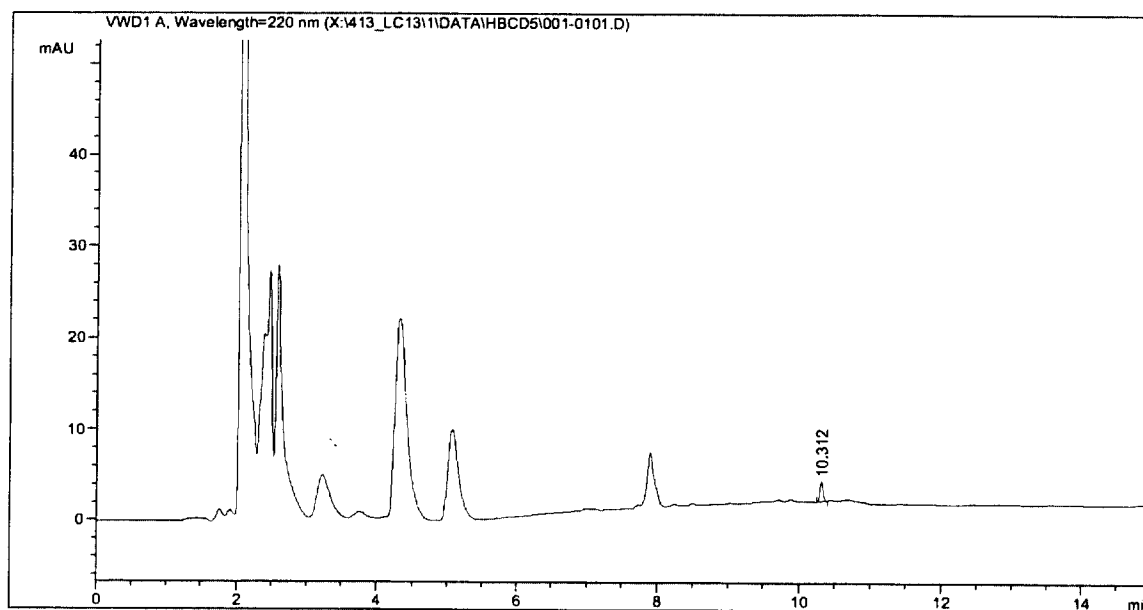
Representative Calibration Curve for Hexabromocyclododecane (HBCD) for Sediment Analysis



Slope = 7.62; Y-Intercept = -0.1538; $R^2 = 0.9999$

APPENDIX 6.8

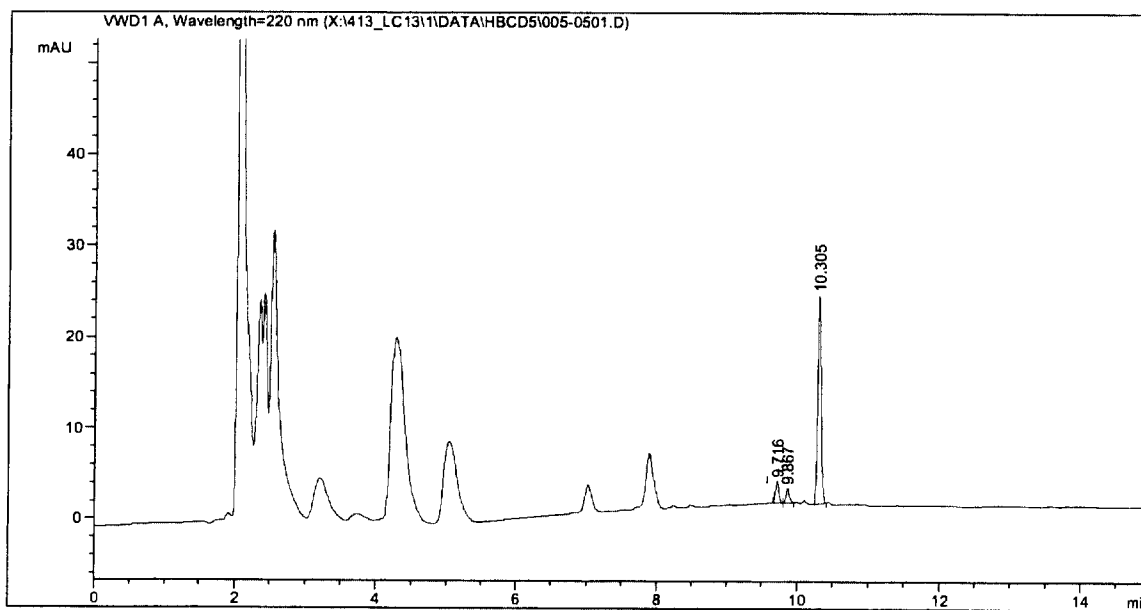
Chromatogram of a Low-level Hexabromocyclododecane (HBCD) Calibration Standard for Sediment Analysis



Nominal concentration: 1.00 mg/L

APPENDIX 6.9

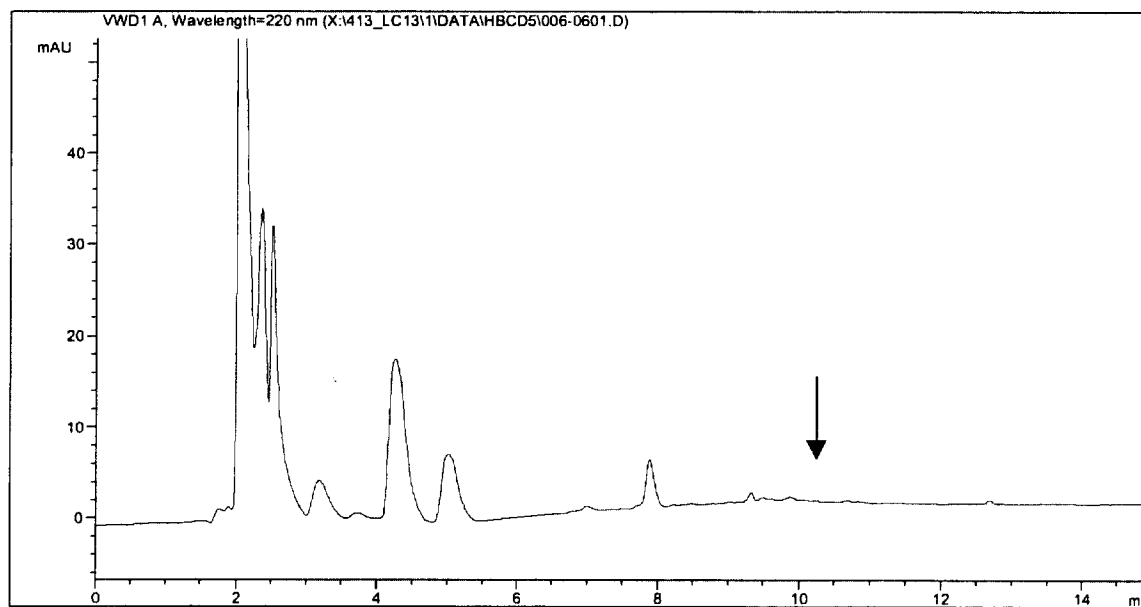
Chromatogram of a High-level Hexabromocyclododecane (HBCD) Calibration Standard for Sediment Analysis



Nominal concentration: 10.0 mg/L

APPENDIX 6.10

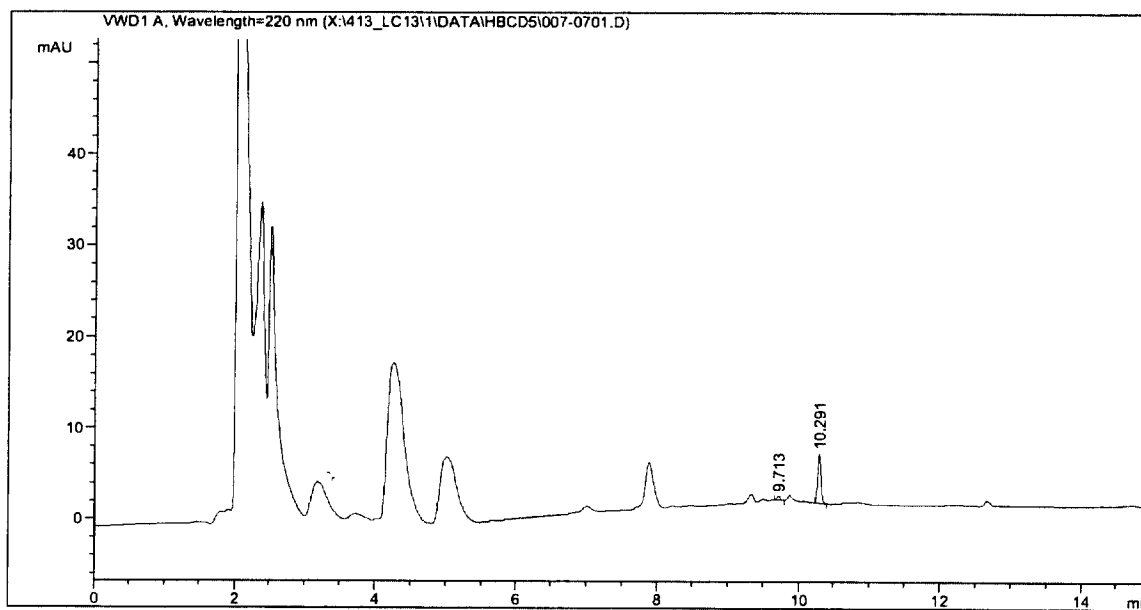
Chromatogram of a Matrix Blank for Sediment



Sample number 439A-120-SMAB-1. Arrow indicates the approximate retention time of Hexabromocyclododecane (HBCD).

APPENDIX 6.11

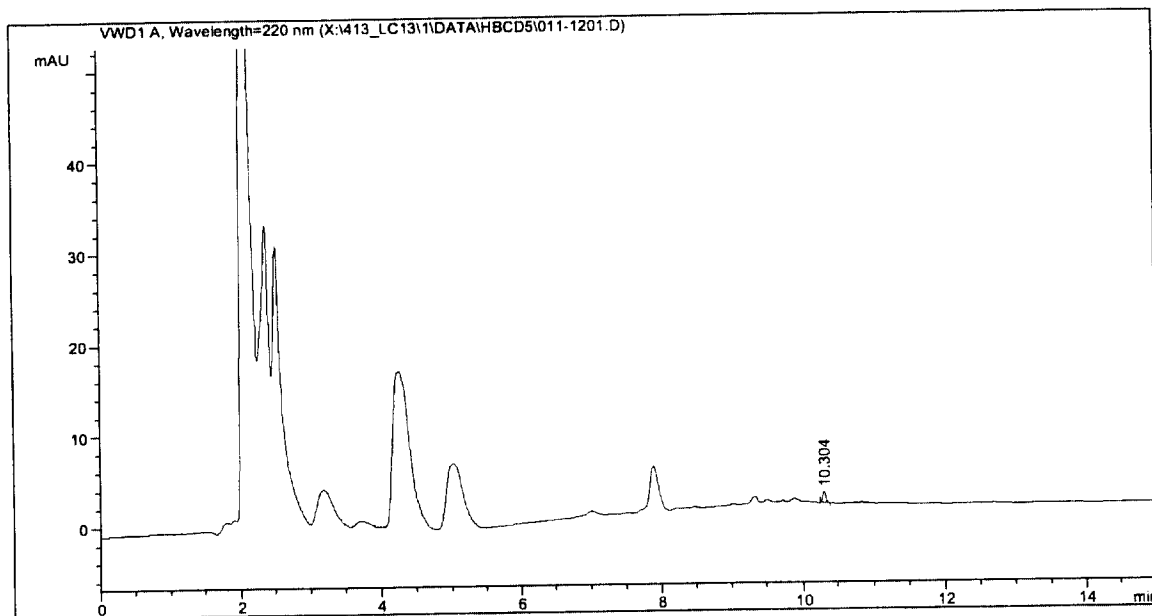
Chromatogram of a Matrix Fortification in Sediment



Sample number 439A-120-SMAS-30.0 µg/g, nominal concentration.

APPENDIX 6.12

Chromatogram of a Day 0 Sediment Sample



Sample number 439A-120-S3; 31 µg/g nominal concentration.

APPENDIX 6.13

**Analytical Method Flowchart for the Analysis of Hexabromocyclododecane (HBCD)
in Aqueous Solutions**

**METHOD OUTLINE FOR THE ANALYSIS OF
HEXABROMOCYCLODODECANE (HBCD) IN AQUEOUS SOLUTIONS**

Prepare each quality control (QC) sample by adding 50 mL of well water to a 125-mL separatory funnel. Fortify each QC sample with the appropriate HBCD stock solution. The matrix blank sample will not be fortified. Add 25 mL of dichloromethane (DCM) to each separatory funnel



Transfer 50 mL of each study test solution directly into a separatory funnel containing 25 mL of dichloromethane (DCM).



Shake the QC and study sample solutions (with venting) for approximately one minute and allow the two phases to separate.



For each sample, drain the organic (lower) phase into a 125-mL roundbottom flask. Add a second 25-mL aliquot of DCM to the aqueous phase remaining in the funnel and perform a second extraction.



For each sample, combine the organic phase from the second extraction with the organic phase of the first extraction in the same roundbottom flask.



Rotary evaporate the organic extracts to 1-2 mL under vacuum and a waterbath set at approximately 40°C.



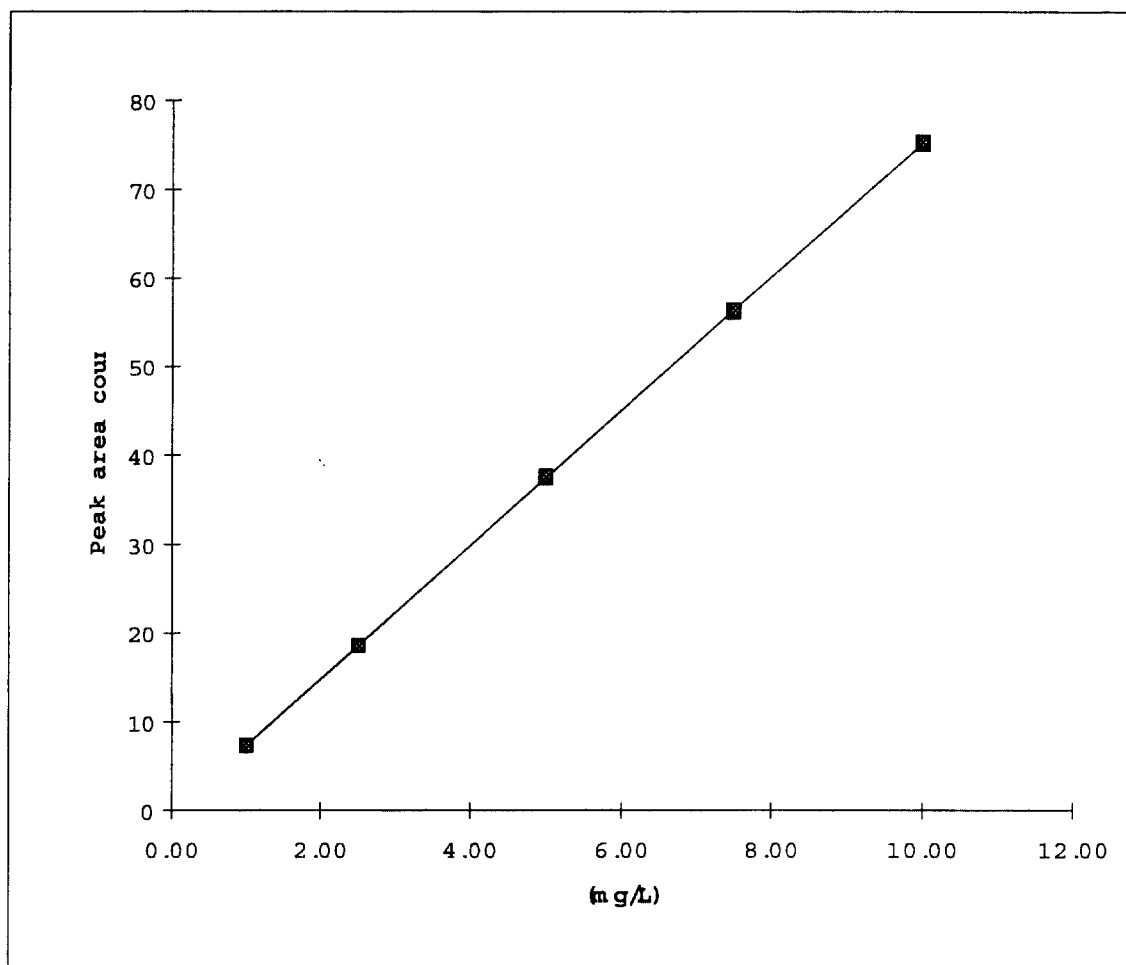
Evaporate the residual DCM in each flask to dryness under a gentle stream of nitrogen. Reconstitute the residues with an appropriate volume of 50% THF : 50% water.



Transfer an aliquot of the diluted extracts into an autosampler vial. Submit for HPLC/UV analysis.

APPENDIX 6.14

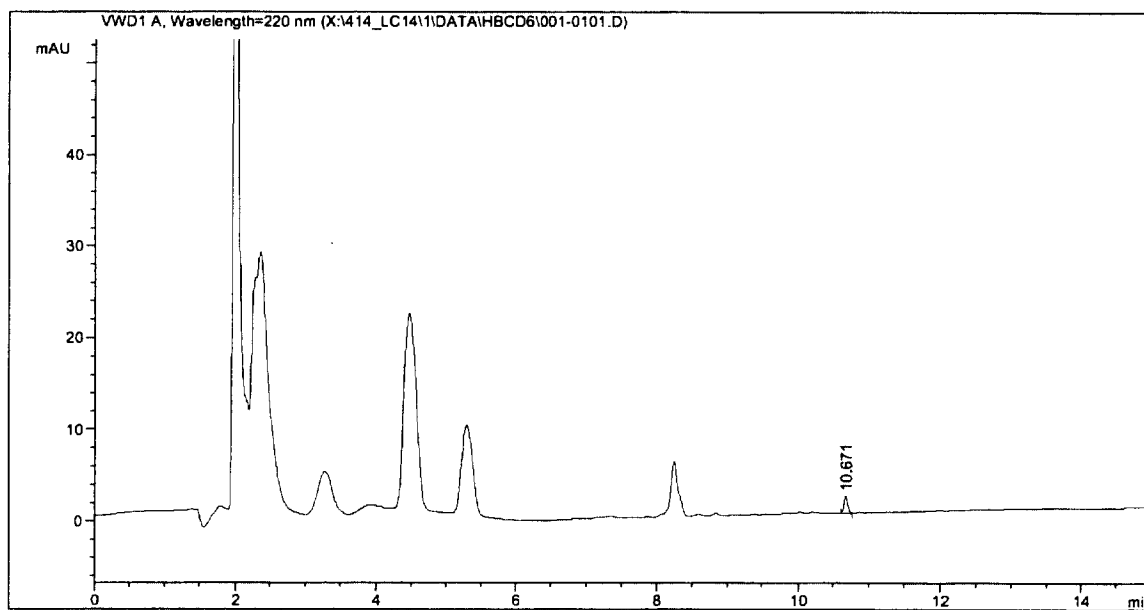
Representative Calibration Curve for Hexabromocyclododecane (HBCD) for Freshwater Analysis



Slope = 7.54; Y-Intercept = -0.1815; $R^2 = 1.0000$

APPENDIX 6.15

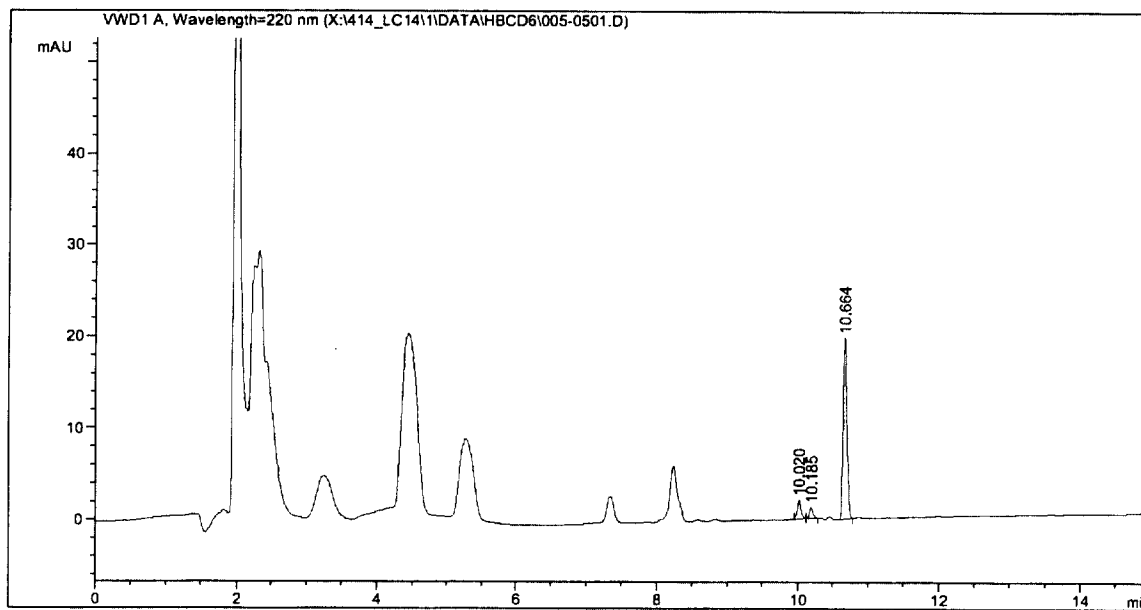
Chromatogram of a Low-level Hexabromocyclododecane (HBCD) Calibration Standard for
Freshwater Analysis



Nominal concentration: 1.00 mg/L

APPENDIX 6.16

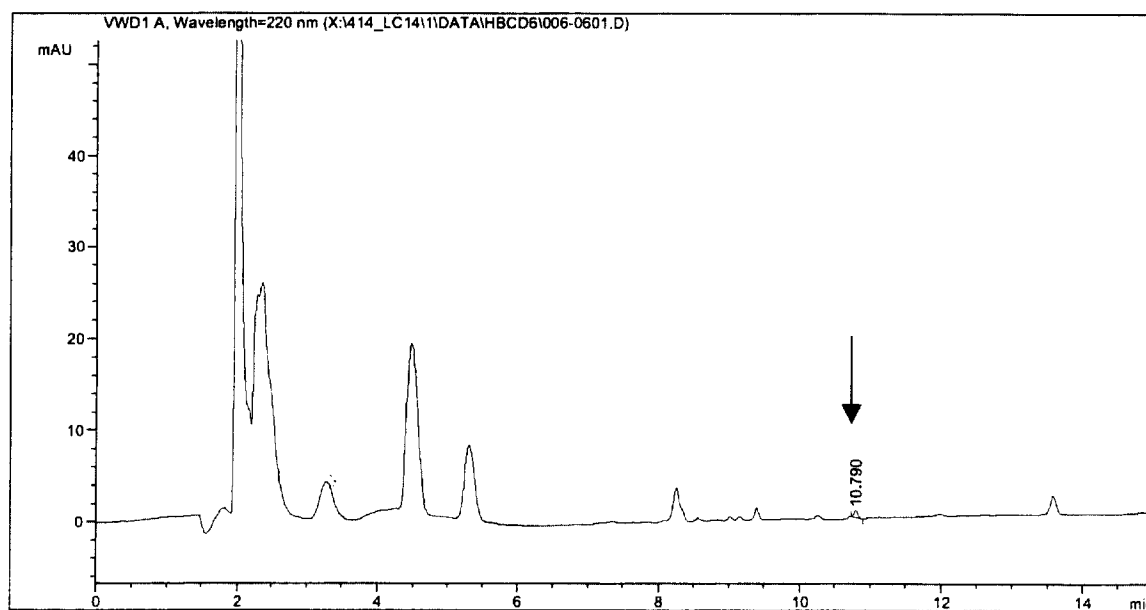
**Chromatogram of a High-level Hexabromocyclododecane (HBCD) Calibration Standard for
Freshwater Analysis**



Nominal concentration: 10.0 mg/L

APPENDIX 6.17

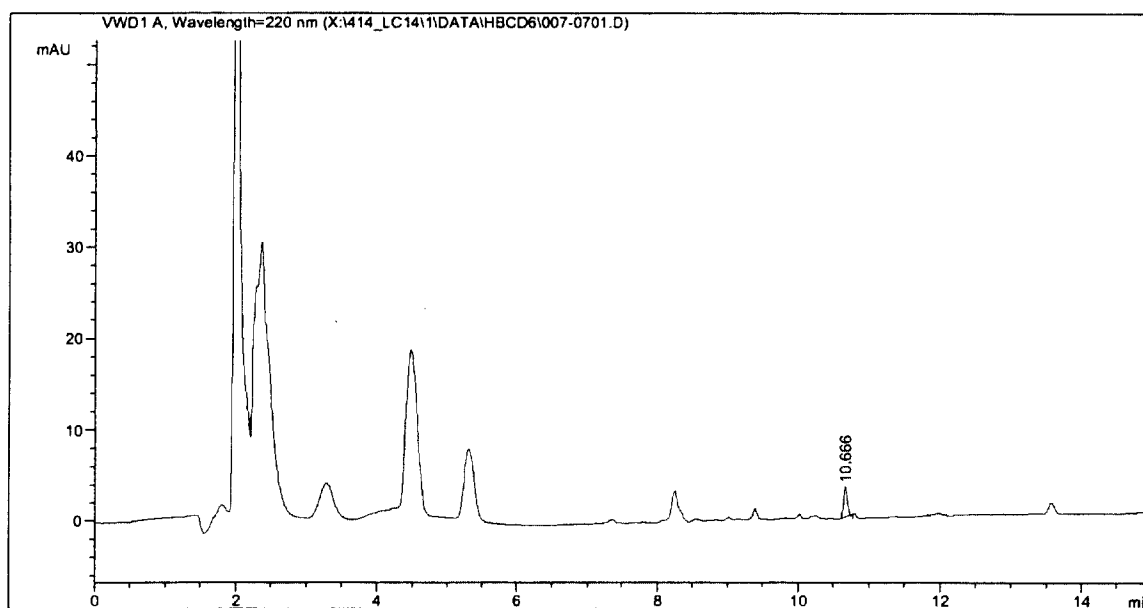
Chromatogram of a Matrix Blank for Freshwater



Sample number 439A-120-MAB-1. Arrow indicates the approximate retention time of Hexabromocyclododecane (HBCD).

APPENDIX 6.18

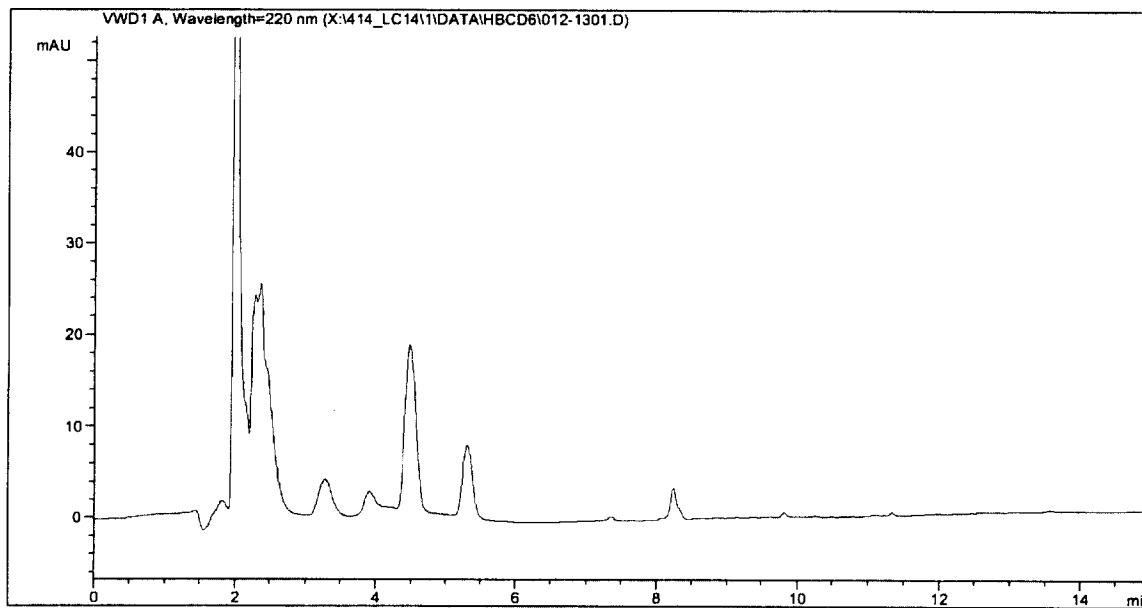
Chromatogram of a Matrix Fortification in Freshwater



Sample number 439A-120-MAS-1; 0.200 mg/L nominal concentration.

APPENDIX 6.19

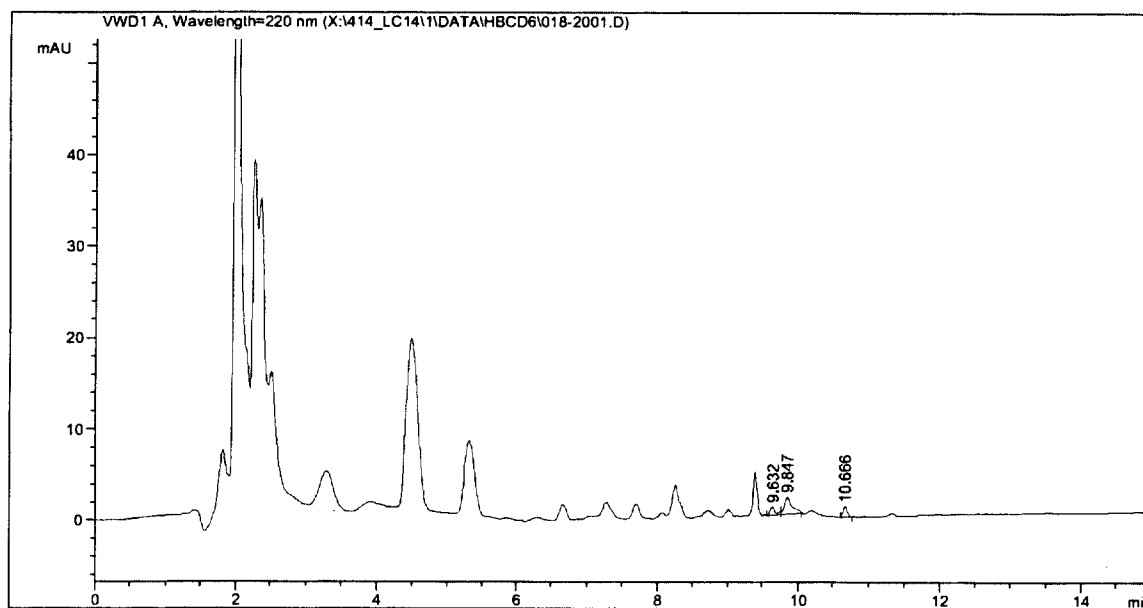
Chromatogram of a Day 0 Overlying Water Sample



Sample number 439A-120-W4; 31 µg/g nominal concentration.

APPENDIX 6.20

Chromatogram of a Day 0 Pore Water Sample



Sample number 439A-120-PW4; 31 µg/g nominal concentration.

APPENDIX 7

Observations of Mortality and Effects by Replicate in a 28-Day
Sediment Toxicity Test with the Amphipod *Hyaella azteca*

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APPENDIX 7
Page 1Observations of Mortality and Effects by Replicate in a 28-Day Sediment Toxicity Test with the Amphipod *Hyalella azteca*

Nominal Concentration (mg/Kg)	Replicate	Number Exposed	Day 0		Day 1		Day 2		Day 5		Day 7		Day 9	
			No. Dead	Obs. ¹	No. Dead	Obs.	No. Dead	Obs.	No. Dead	Obs.	No. Dead	Obs.	No. Dead	Obs.
Negative Control	A	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	B	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	C	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	D	10	0	AN	1	AN	0	AN	0	AN	0	AN	0	AN
	E	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	F	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	G	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	H	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
31	A	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	B	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	C	10	0	AN	1	AN	0	AN	0	AN	0	AN	0	AN
	D	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	E	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	F	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	G	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	H	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN

¹ Observations: AN = appear normal.

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APPENDIX 7 (continued)

Page 2

Observations of Mortality and Effects by Replicate in a 28-Day Sediment Toxicity Test with the Amphipod *Hyalella azteca*

Nominal Concentration (mg/Kg)	Replicate	Number Exposed	Day 0		Day 1		Day 2		Day 5		Day 7		Day 9	
			No. Dead	Obs. ¹	No. Dead	Obs.	No. Dead	Obs.	No. Dead	Obs.	No. Dead	Obs.	No. Dead	Obs.
63	A	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	B	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	C	10	0	AN	1	AN	0	AN	0	AN	0	AN	0	AN
	D	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	E	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	F	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	G	10	0	AN	1	AN	0	AN	0	AN	0	AN	0	AN
	H	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
125	A	10	0	AN	2	AN	0	AN	0	AN	0	AN	0	AN
	B	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	C	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	D	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	E	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	F	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	G	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	H	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN

¹ Observations: AN = appear normal.

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APPENDIX 7 (continued)

Page 3

Observations of Mortality and Effects by Replicate in a 28-Day Sediment Toxicity Test with the Amphipod *Hyalella azteca*

Nominal Concentration (mg/Kg)	Replicate	Number Exposed	Day 0		Day 1		Day 2		Day 5		Day 7		Day 9	
			No. Dead	Obs. ¹	No. Dead	Obs.	No. Dead	Obs.	No. Dead	Obs.	No. Dead	Obs.	No. Dead	Obs.
250	A	10	0	AN	1	AN	0	AN	0	AN	0	AN	0	AN
	B	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	C	10	0	AN	1	AN	0	AN	0	AN	0	AN	0	AN
	D	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	E	10	1	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	F	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	G	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	H	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
500	A	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	B	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	C	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	D	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	E	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	F	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	G	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	H	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN

¹ Observations: AN = appear normal.

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APPENDIX 7 (continued)

Page 4

Observations of Mortality and Effects by Replicate in a 28-Day Sediment Toxicity Test with the Amphipod *Hyalella azteca*

Nominal Concentration (mg/kg)	Replicate	Number Exposed	Day 0		Day 1		Day 2		Day 5		Day 7		Day 9	
			No. Dead	Obs. ¹	No. Dead	Obs.	No. Dead	Obs.	No. Dead	Obs.	No. Dead	Obs.	No. Dead	Obs.
1000	A	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	B	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	C	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	D	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	E	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	F	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	G	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN
	H	10	0	AN	0	AN	0	AN	0	AN	0	AN	0	AN

¹ Observations: AN = appear normal.

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APPENDIX 7 (continued)

Page 5

Observations of Mortality and Effects by Replicate in a 28-Day Sediment Toxicity Test with the Amphipod (*Hyalella azteca*)

Nominal Concentration (mg/Kg)	Replicate	Day 12		Day 14		Day 16		Day 19		Day 21		Day 23	
		No. Dead	Obs. ¹	No. Dead	Obs.	No. Dead	Obs.	No. Dead	Obs.	No. Dead	Obs.	No. Dead	Obs.
Negative Control	A	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN	0	AN ²
	B	0	AN ²	0	AN	0	AN ²	0	AN ²	0	AN	0	AN ²
	C	0	AN ²	0	AN	0	AN ²	0	AN ²	0	AN	0	AN ²
	D	0	AN ²	0	AN	0	AN ²	0	AN ²	0	AN	0	AN ²
	E	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN	0	AN ²
	F	0	AN ²	0	AN	0	AN ²	0	AN ²	0	AN	0	AN ²
	G	0	AN ²	0	AN	0	AN ²	0	AN ²	0	AN	0	AN ²
	H	0	AN ²	0	AN	0	AN ²	0	AN ²	0	AN	0	AN ²
31	A	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²
	B	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²
	C	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²
	D	0	AN ²	0	AN ²	0	AN	0	AN ²	0	AN ²	0	AN ²
	E	0	AN ²	0	AN	0	AN ²	0	AN ²	0	AN ²	0	AN ²
	F	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²
	G	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²
	H	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²

¹ Observations: AN = appear normal.² Fungal growth present in this test compartment.

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APPENDIX 7 (continued)

Page 6

Observations of Mortality and Effects by Replicate in a 28-Day Sediment Toxicity Test with the Amphipod *Hyalella azteca*

Nominal Concentration (mg/Kg)	Replicate	Day 12		Day 14		Day 16		Day 19		Day 21		Day 23	
		No. Dead	Obs. ¹	No. Dead	Obs.	No. Dead	Obs.	No. Dead	Obs.	No. Dead	Obs.	No. Dead	Obs.
63	A	0	AN ²	0	AN	0	AN ²	0	AN ²	0	AN ²	0	AN ²
	B	0	AN ²	0	AN	0	AN ²	0	AN ²	0	AN ²	0	AN ²
	C	0	AN ²	0	AN	0	AN ²	0	AN ²	0	AN ²	0	AN ²
	D	0	AN ²	0	AN	0	AN ²	0	AN ²	0	AN ²	0	AN ²
	E	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²
	F	0	AN ²	0	AN	0	AN ²	0	AN ²	0	AN ²	0	AN ²
	G	0	AN ²	0	AN	0	AN ²	0	AN ²	0	AN ²	0	AN ²
	H	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²
125	A	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²
	B	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²
	C	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²
	D	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²
	E	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²
	F	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²
	G	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²
	H	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²

¹ Observations: AN = appear normal.² Fungal growth present in this test compartment.

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APPENDIX 7 (continued)

Page 7

Observations of Mortality and Effects by Replicate in a 28-Day Sediment Toxicity Test with the Amphipod *Hyalella azteca*

Nominal Concentration (mg/Kg)	Replicate	Day 12		Day 14		Day 16		Day 19		Day 21		Day 23	
		No. Dead	Obs. ¹	No. Dead	Obs.	No. Dead	Obs.	No. Dead	Obs.	No. Dead	Obs.	No. Dead	Obs.
250	A	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²
	B	0	AN	0	AN	0	AN ²	0	AN ²	0	AN ²	0	AN ²
	C	0	AN	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²
	D	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²
	E	0	AN	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²
	F	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²
	G	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²
	H	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²
500	A	0	AN ²	0	AN	0	AN ²	0	AN ²	0	AN ²	0	AN ²
	B	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²
	C	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²
	D	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²
	E	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²
	F	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²
	G	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²
	H	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²

¹ Observations: AN = appear normal.² Fungal growth present in this test compartment.

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APPENDIX 7 (continued)
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Observations of Mortality and Effects by Replicate in a 28-Day Sediment Toxicity Test with the Amphipod *Hyalella azteca*

Nominal Concentration (mg/Kg)	Replicate	Day 12		Day 14		Day 16		Day 19		Day 21		Day 23	
		No. Dead	Obs. ¹	No. Dead	Obs.	No. Dead	Obs.	No. Dead	Obs.	No. Dead	Obs.	No. Dead	Obs.
1000	A	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²
	B	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²
	C	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²
	D	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²
	E	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²
	F	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²
	G	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²
	H	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²	0	AN ²

¹ Observations: AN= appear normal.² Fungal growth present in this test compartment.

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APPENDIX 7 (continued)
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Observations of Mortality and Effects by Replicate in a 28-Day Sediment Toxicity Test with the Amphipod *Hyalella azteca*

Nominal Concentration (mg/Kg)	Replicate	Day 26		Day 27		No. Dead or MAD ³	Day 28		No. Survivors
		No. Dead	Obs. ¹	No. Dead	Obs.		Obs.		
Negative Control	A	0	AN ²	0	AN ²	3	AN ²	7	
	B	0	AN ²	0	AN ²	0	AN ²	10	
	C	0	AN ²	0	AN ²	0	AN ²	10	
	D	0	AN ²	0	AN ²	2	AN ²	8	
	E	0	AN ²	0	AN ²	0	AN ²	10	
	F	0	AN ²	0	AN ²	1	AN ²	9	
	G	0	AN ²	0	AN ²	0	AN ²	10	
	H	0	AN ²	0	AN ²	1	AN ²	9	
						Mean:	9.1		
31	A	0	AN ²	0	AN ²	0	AN ²	10	
	B	0	AN ²	0	AN ²	1	AN ²	9	
	C	0	AN ²	0	AN ²	3	AN ²	7	
	D	0	AN ²	0	AN ²	1	AN ²	9	
	E	0	AN ²	0	AN ²	1	AN ²	9	
	F	0	AN ²	0	AN ²	2	AN ²	8	
	G	0	AN ²	0	AN ²	1	AN ²	9	
	H	0	AN ²	0	AN ²	2	AN ²	8	
						Mean:	8.6		

¹ Observations: AN = appear normal.² Fungal growth present in this test compartment.³ MAD = missing and assumed dead.

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APPENDIX 7 (continued)

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Observations of Mortality and Effects by Replicate in a 28-Day Sediment Toxicity Test with the Amphipod *Hyalella azteca*

Nominal Concentration (mg/Kg)	Replicate	Day 26		Day 27		No. Dead or MAD ³	Day 28	
		No. Dead	Obs. ¹	No. Dead	Obs.		Obs.	No. Survivors
63	A	0	AN ²	0	AN ²	1	AN ²	9
	B	0	AN ²	0	AN ²	5	AN ²	5
	C	0	AN ²	0	AN ²	4	AN ²	6
	D	0	AN ²	0	AN ²	5	AN ²	5
	E	0	AN ²	0	AN ²	5	AN ²	5
	F	0	AN ²	0	AN ²	6	AN ²	4
	G	0	AN ²	0	AN ²	7	AN ²	3
	H	0	AN ²	0	AN ²	0	AN ²	10
							Mean:	5.9
125	A	0	AN ²	0	AN ²	5	AN ²	5
	B	0	AN ²	0	AN ²	3	AN ²	7
	C	0	AN ²	0	AN ²	3	AN ²	7
	D	0	AN ²	0	AN ²	4	AN ²	6
	E	0	AN ²	0	AN ²	3	AN ²	7
	F	0	AN ²	0	AN ²	-- ⁴	--	--
	G	0	AN ²	0	AN ²	3	AN ²	7
	H	0	AN ²	0	AN ²	6	AN ²	4
							Mean:	6.1

¹ Observations: AN = appear normal.² Fungal growth present in this test compartment.³ MAD = missing and assumed dead.⁴ Replicate found empty. All sediment overflowed to test chamber. Accurate count of organisms was not obtained.

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APPENDIX 7 (continued)

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Observations of Mortality and Effects by Replicate in a 28-Day Sediment Toxicity Test with the Amphipod *Hyalella azteca*

Nominal Concentration (mg/Kg)	Replicate	Day 26		Day 27		No. Dead or MAD ³	Day 28	
		No. Dead	Obs. ¹	No. Dead	Obs.		Obs.	No. Survivors
250	A	0	AN ²	0	AN ²	3	AN ²	7
	B	0	AN ²	0	AN ²	1	AN ²	9
	C	0	AN ²	0	AN ²	5	AN ²	5
	D	0	AN ²	0	AN ²	2	AN ²	8
	E	0	AN ²	0	AN ²	4	AN ²	6
	F	0	AN ²	0	AN ²	3	AN ²	7
	G	0	AN ²	0	AN ²	2	AN ²	8
	H	0	AN ²	0	AN ²	4	AN ²	6
							Mean:	7.0
500	A	0	AN ²	0	AN ²	7	AN ²	3
	B	0	AN ²	0	AN ²	0	AN ²	10
	C	0	AN ²	0	AN ²	1	AN ²	9
	D	0	AN ²	0	AN ²	1	AN ²	9
	E	1	AN ²	0	AN ²	0	AN ²	10
	F	0	AN ²	0	AN ²	1	AN ²	9
	G	0	AN ²	0	AN ²	2	AN ²	8
	H	0	AN ²	0	AN ²	0	AN ²	10
							Mean:	8.5

¹ Observations: AN = appear normal.² Fungal growth present in this test compartment.³ MAD = missing and assumed dead.

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APPENDIX 7 (continued)

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Observations of Mortality and Effects by Replicate in a 28-Day Sediment Toxicity Test with the Amphipod *Hyalella azteca*

Nominal Concentration (mg/Kg)	Replicate	Day 26		Day 27		No. Dead or MAD ³	Day 28	
		No. Dead	Obs. ¹	No. Dead	Obs.		Obs.	No. Survivors
1000	A	0	AN ²	0	AN ²	1	AN ²	9
	B	0	AN ²	0	AN ²	2	AN ²	8
	C	0	AN ²	0	AN ²	1	AN ²	9
	D	0	AN ²	0	AN ²	0	AN ²	10
	E	0	AN ²	0	AN ²	1	AN ²	9
	F	0	AN ²	0	AN ²	2	AN ²	8
	G	0	AN ²	0	AN ²	0	AN ²	10
	H	0	AN ²	0	AN ²	0	AN ²	10
							Mean:	9.1

¹ Observations: AN = appear normal.² Fungal growth present in this test compartment.³ MAD = missing and assumed dead.

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APPENDIX 8

Amphipod Dry Weights by Replicate in a 28-Day Sediment
Toxicity Test with the Amphipod *Hyaella azteca*

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APPENDIX 8

Amphipod Dry Weights by Replicate in a 28-Day Sediment Toxicity Test with the Amphipod *Hyalella azteca*

Replicate	Average Individual Dry Weight (mg) by Nominal Concentration						
	Negative Control	31 (mg/Kg)	63 (mg/Kg)	125 (mg/Kg)	250 (mg/Kg)	500 (mg/Kg)	1000 (mg/Kg)
A	0.13	0.19	0.22	0.18	0.27	0.13	0.20
B	0.23	0.18	0.32	0.21	0.19	0.24	0.24
C	0.18	0.13	0.20	0.16	0.18	0.23	0.20
D	0.13	0.20	0.28	0.17	0.18	0.18	0.13
E	0.22	0.18	0.32	0.24	0.15	0.18	0.19
F	0.24	0.16	0.23	-- ¹	0.24	0.19	0.16
G	0.16	0.13	0.20	0.37	0.21	0.19	0.19
H	0.26	0.21	0.31	0.20	0.17	0.15	0.20
Mean ± Std. Dev.	0.19 ± 0.05	0.17 ± 0.03	0.26 ± 0.05	0.22 ± 0.07	0.20 ± 0.04	0.19 ± 0.04	0.19 ± 0.03

¹ Replicate was compromised, therefore dry weights could not be determined.

¹ Replicate was compromised, therefore dry weights could not be determined.

APPENDIX 9

HBCD Non-GLP Rangefinding Results

APPENDIX 9

**HBCD Non-GLP Rangefinding Results
Oligochaete**

2% organic carbon

SURVIVAL			
mg/kg	Rep A n=10	Rep B n=10	Total
NC	13	20	33
50	27	39	66
100	33	23	56
500	21	28	49
1000	31	21	52

Mean Individual Dry Weight			
mg/kg	Rep A n=10	Rep B n=10	Mean
NC	2.24	1.61	1.925
50	1.88	1.53	1.705
100	1.05	1.48	1.265
500	1.59	1.15	1.37
1000	1.75	2.08	1.915

5% organic carbon

SURVIVAL			
mg/kg	Rep A n=10	Rep B n=10	Total
NC	34	35	69
50	23	24	47
100	33	32	65
500	23	24	47
1000	23	27	50

Mean Individual Dry Weight			
mg/kg	Rep A n=10	Rep B n=10	Mean
NC	1.39	0.88	1.135
50	1.25	1.47	1.36
100	1.19	1.33	1.26
500	1.23	1.11	1.17
1000	1.87	1.02	1.445

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APPENDIX 9 (continued)**HBCD Non-GLP Rangefinding Results
Chironomid****5% organic carbon**

mg/kg	Rep A n=20	Rep B n=20	Total	% Emergence
NC	19	18	37	92.5
50	8	16	24	60
100	20	18	38	95
500	14	19	33	82.5
1000	20	14	34	85

2% organic carbon

mg/kg	Rep A n=20	Rep B n=20	Total	% Emergence
NC	10	13	23	57.5
50	20	20	40	100
100	16	10	26	65
500	20	15	35	87.5
1000	6	20	26	65

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APPENDIX 9 (continued)**HBCD Non-GLP Rangefinding Results
Hyaella****2% organic carbon**

mg/kg	Rep A n=10	% Survival
NC	8	80
50	8	80
100	9	90
500	6	60
1000	4	40

5% organic carbon

mg/kg	Rep A n=10	% Survival
NC	9	90
50	8	80
100	8	80
500	4	40
1000	1	10

APPENDIX 10

Personnel Involved in the Study

The following key Wildlife International, Ltd. personnel were involved in the conduct or management of this study:

1. Mark Jaber, Director of Research
2. Henry O. Krueger, Ph.D., Director of Aquatic Toxicology/Terrestrial Plants and Insects
3. Willard B. Nixon, Ph.D., Director of Chemistry
4. Timothy Z. Kendall, Laboratory Supervisor
5. Cary A. Sutherland, Laboratory Supervisor
6. Susan Thomas, Biologist
7. Tim Ross, Biologist
8. Amy Blankinship, Biologist

STUDY TITLE

HEXABROMOCYCLODODECANE: CONTACT SENSITIZATION POTENTIAL
VIA THE LOCAL LYMPH NODE ASSAY (INCLUDING A PRIMARY IRRITANCY
SCREEN) USING CBA/J MICE

Data Requirement

U.S. EPA OPPTS Guideline No. 870.2600
OECD Guideline No. 429

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Study Completion Date

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Performing Laboratory

Toxicology & Environmental Research and Consulting
The Dow Chemical Company
Midland, Michigan 48674

Laboratory Project Study ID

031013

COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

Compound: HEXABROMOCYCLODODECANE

Title: HEXABROMOCYCLODODECANE: CONTACT SENSITIZATION
POTENTIAL VIA THE LOCAL LYMPH NODE ASSAY (INCLUDING
A PRIMARY IRRITANCY SCREEN) USING CBA/J MICE

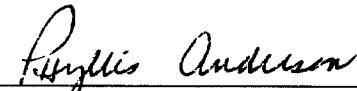
With one exception, all phases of this study were conducted in compliance with the Good Laboratory Practice Standards listed below. The concentrations of the dosing solutions prepared by Immunotoxicology personnel for the primary irritancy screen and the LLNA were not verified analytically.

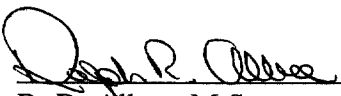
Japanese Ministry of International Trade and Industry (MITI)
Good Laboratory Practice Standards Applied to Industrial Chemicals

US Environmental Protection Agency - TSCA GLPs
Title 40 CFR, Part 792 - Toxic Substances Control Act (TSCA); Good Laboratory
Practice Standards, Final Rule

Organisation for Economic Co-Operation and Development (OECD)
OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring,
Number 1. OECD Principles on Good Laboratory Practice (as revised in 1997)
ENV/MC/CHEM(98)17

European Community (EC)
EC Directive 99/11/EC of 8 March 1999 (OJ No. L 77/8-21, 23/3/1999)


P. K. Anderson, A.S. (Date) 7-14-03
Study Director


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Research and Consulting

QUALITY ASSURANCE STATEMENT

Compound: HEXABROMOCYCLODODECANE

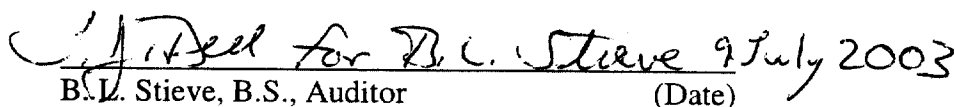
Title: HEXABROMOCYCLODODECANE: CONTACT SENSITIZATION
POTENTIAL VIA THE LOCAL LYMPH NODE ASSAY (INCLUDING
A PRIMARY IRRITANCY SCREEN) USING CBA/J MICE

This study was examined for conformance with Good Laboratory Practices as published by the USEPA (TSCA), MITI, OECD, and EC. The final report was determined to be an accurate reflection of the data obtained. The dates of Quality Assurance activities on this study are listed below.

Study Initiation Date: 8-April-03

<u>TYPE OF AUDIT:</u>	<u>DATE OF AUDIT:</u>	<u>DATE FINDINGS REPORTED TO STUDY DIRECTOR/MANAGEMENT:</u>
Final protocol	24-April-03	24-April-03
Study conduct	25-April-03	25-April-03
Protocol, data, and draft report	03-June-03	05-June-03
Final report	The date of the signature below is the date of the final report audit.	

The final report accurately reflects the raw data of the study.

 for B.L. Stieve 9 July 2003
(Date)

B.L. Stieve, B.S., Auditor
Quality Assurance
Toxicology & Environmental Research and Consulting
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SUMMARY

The Local Lymph Node Assay (LLNA) assesses the potential of test materials to cause contact sensitization by measuring the lymphocyte proliferative responses from auricular lymph nodes following topical application of the test materials to mouse ears. Test materials that elicit a Stimulation Index (SI) of ≥ 3 (*i.e.*, 3- fold greater proliferation than control animals) should be considered positive for dermal sensitization potential.

All mice received one of three concentrations of HBCD (2%, 20% or 50% w/v) or DMF (dimethylformamide) on days 1-3 (n=6 mice/group). HCA (α -hexyl cinnamaldehyde), a moderate contact sensitizer, was evaluated concurrently as a positive dermal sensitization control. The test materials were administered to the dorsal surface of both ears (25 μ l/ear). On day 6, all mice received an intravenous tail vein injection of phosphate buffered saline containing 20 μ Ci of 3 H-thymidine. Uptake into the auricular lymph nodes draining the site of chemical application was measured 5 hours later.

Body weight data were unremarkable and minor increases in ear thickness were noted suggesting slight irritation following applications of 20% and 50% HBCD. There were no indications that HBCD possesses dermal sensitization potential. SI values were consistently around 1.0 at all doses tested. Lymphocyte proliferation by DMF, vehicle treated mice (2015 dpm) was higher than historical laboratory values commonly observed using acetone and olive oil as a vehicle. This is not inconsistent with that reported in the literature for this vehicle. HCA administrations (30% v/v) elicited proliferation that was 3-fold greater than that of vehicle controls thus detecting the moderate contact sensitization potential in this study. On the basis of these results, HBCD would not be considered to have contact sensitization potential.

INTRODUCTION

Purpose

The Local Lymph Node Assay (LLNA) assesses the potential of test materials to cause contact sensitization by measuring the lymphocyte proliferative responses from auricular lymph nodes following topical application of the test materials to mouse ears. Previous studies have determined that chemicals with irritancy potential, but without sensitization potential, can elicit lymphocyte proliferation when administered at concentrations that induce irritation to mouse ears. Thus, mouse ear swelling was evaluated to identify potentially confounding test material concentrations that produce irritation to the ears of mice. Mouse ear thickness was used as an indication of ear irritation and is only relevant in the context of the LLNA, and should not be interpreted as an indication of irritation potential in humans. Sensitization potential was subsequently determined by the magnitude of lymphocyte proliferative response in the auricular lymph nodes draining the ears. Test materials that elicit a Stimulation Index (SI) of ≥ 3 (*i.e.*, 3- fold greater proliferation than control animals) should be considered positive for dermal sensitization potential.

Quality Assurance

The study conduct, data, protocol, protocol changes/revisions, and final report were inspected by the Quality Assurance Unit, Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan.

Archiving

The data, protocol, protocol changes/revisions, and final report are archived at Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan.

TEST MATERIAL INFORMATION

Test Material Chemical Name

1,2,5,6,9,10-Hexabromocyclododecane

Test Material Synonym

HBCD

Supplier, City, State (reference number)

The test article was received from Wildlife International, Ltd. (Easton, Maryland) on February 24, 2003 and was a composite of three manufacturers' HBCD commercial product (Wildlife International test substance no. 5850):

Albemarle Corporation
Baton Rouge, Louisiana 70801

Dead Sea Bromine Group
Beer Sheva, Israel

Great Lakes Chemical Corporation
West Lafayette, Indiana 47996

Purity/Characterization (method of analysis and reference)

Purity and characterization was conducted according to GLP conditions by the laboratories of Albemarle Corporation. The identity of HBCD was confirmed by Fourier Transform Infrared Spectroscopy while the homogeneity of the test material was determined to be greater than 95% using HPLC analysis. The HBCD test material was comprised of three diastereomers: 5.8% alpha, 19.3% beta, and 74.9% gamma.

Characteristics

Appearance (physical state, color)

White/powder

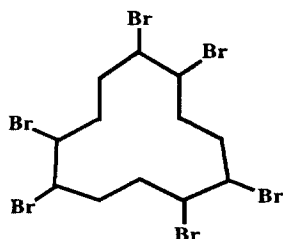
Molecular Formula

$C_{12}H_{18}Br_6$

Molecular Weight

641.7

Chemical Structure



Previous Toxicity Information

No health hazards have been identified on the manufacturer's MSDS. HBCD has been reported to not be acutely toxic or irritating.

TEST ANIMALS

Species and Sex

Mice (female)

Supplier and Location

The Jackson Laboratory, Bar Harbor, Maine

Strain and Justification

CBA/J mice are being utilized in accordance with OPPTS and OECD guidelines that outline conduct of the mouse LLNA.

Animal Welfare

In response to the Final Rules amending the U.S. Animal Welfare Act promulgated by the U.S. Department of Agriculture effective October 30, 1989, the Animal Care and Use Activities (ACUA) required for the conduct of this study were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC). The IACUC determined that the proposed Activities were in full accordance with these Final Rules. The IACUC assigned File No. Immun 03 and Animal ID 01 to these Animal Care and Use Activities.

Physical and Acclimation

Each animal was evaluated by a laboratory veterinarian or a trained animal/toxicology technician, under the direct supervision of a lab veterinarian to determine their general health status and acceptability for study purposes upon arrival at the laboratory¹. The animals were housed in plastic "shoebox" cages, in rooms designed to maintain adequate conditions (temperature, humidity, and photocycle), and acclimated to the laboratory for approximately seven days prior to the start of the study.

¹ Fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International).

Housing

The animals (6/group) were housed in plastic "shoebox" cages with filter lids in rooms designed to maintain adequate conditions (temperature, humidity, and photocycle), and acclimated to the laboratory for approximately one week prior to the start of the study. Each cage contained corncob bedding and a cardboard enclosure, which were changed at least two times a week. Food pellets and a water bottle with a stainless steel sipper tube were suspended above the mice using a wire rack. The relative humidity and temperature in the room were maintained within a range of 40-70% and 22 ± 1 °C (with a maximum permissible excursion range of ± 3 °C), respectively. These values are within the recommended ranges for housing mice (National Research Council, Guide for the Care and Use of Laboratory Animals, 1996). A 12-hour light/dark photocycle was maintained for the animal rooms with lights on at 6:00 a.m. and off at 6:00 p.m., and room air was exchanged approximately 12-15 times/hour.

Age

The mice were 8 weeks of age at the start of the primary irritancy study and 9 weeks of age at the start of the LLNA.

Randomization and Identification

Animals were stratified using pre-exposure body weights and randomly assigned to treatment groups using a computer program (Provantis created by Instem Corporation, Philadelphia, Pennsylvania). Animals placed on study were uniquely identified via subcutaneously implanted transponders (BioMedic Data Systems, Seaford, Delaware) which are correlated to unique alphanumeric identification numbers.

Feed and Water

Animals were provided LabDiet® Certified Rodent Diet #5002 (PMI Nutrition International, St. Louis, Missouri) in pellet form. Feed and municipal water was provided *ad libitum*. Analysis of the feed was performed by PMI Nutrition International to confirm the diet provides adequate nutrition and to quantify the levels of selected contaminants. Drinking water obtained from the municipal water source is periodically analyzed for chemical parameters and biological contaminants by the municipal water department. In addition, specific analyses for chemical contaminants have been conducted at periodic intervals by an independent testing facility. Copies of these

analyses are maintained at Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan.

STUDY DESIGN

Route Justification

Dermal exposure is considered to be the primary route of induction by chemical contact sensitizers, and as such, was utilized in the LLNA to predict contact sensitization potential.

Ear Swelling Response

Prior to the administration of a dosing solution on the ear and on day 3 of the screening phase of the study, the thickness of each ear was measured with a digital micrometer. The administration of the materials (25 µl per ear) was made for two consecutive days using an adjustable pipettor with a disposable tip (n=1 mice/group). Each mouse received one concentration of either HBCD in vehicle (N,N-dimethylformamide- DMF) or DMF alone on the dorsal surface of each ear. HBCD was not adequately soluble in 4:1 acetone:olive oil (AOO), the preferred vehicle for the LLNA. The highest concentration of HBCD (50% w/v) was based upon maximal solubility of HBCD in DMF; lower concentrations were selected to provide a range of doses (25%, 10%, 5%, or 1%) to evaluate ear swelling potential of HBCD. Ear swelling and irritation are only relevant in the context of the LLNA and should not be used to classify a chemical's irritation potential.

Local Lymph Node Assay

Since mild irritation was noted during the irritation screen, the thickness of each ear was monitored during the LLNA procedure. Prior to the administration of a dosing material and after the final exposure on day 3, the thickness of each ear was measured with a digital micrometer.

The administration of the materials (25 µl/ear) was made on the dorsal surface of both ears using an adjustable pipettor fitted with a disposable tip (n=6 mice/group). All mice received one of three concentrations of HBCD (2%, 20%, or 50%) or DMF on days 1-3. HCA (α-hexyl cinnamaldehyde) at 30% was run concurrently as a positive dermal sensitization control. On day 6, all mice received an intravenous injection (i.v.), via the

lateral tail vein, of 250 µl of phosphate buffered saline (PBS) containing 20 µCi of ³H-thymidine (specific activity 2Ci/mmol; Amersham code TRA310).

Five hours later, the mice were sacrificed and the draining auricular lymph nodes excised and pooled for each mouse. A single cell suspension of lymph node cells (LNC) was prepared by gentle mechanical disaggregation using a tissue homogenizer (Stomach 80 Lab System, Seward Medical Unlimited, London, U.K.). The cells were washed two times in PBS and were precipitated in 5% trichloroacetic acid. ³H-thymidine incorporation was measured on a beta-scintillation counter as disintegration per minute (dpm) per mouse, and a mean dpm value ± SD (standard deviation) was calculated for each experimental group. In addition, a SI was calculated using the absolute dpm value for each mouse as the numerator, and the mean dpm value from the vehicle control mice as the denominator. A mean SI ± SD was calculated for each experimental group. Chemicals that elicit a SI of ≥ 3 in the LLNA are considered positive for dermal sensitization potential.

Clinical Observations

The initial and terminal body weights were obtained and recorded. Once each day a cage-side examination was conducted by animal care personnel, and to the extent possible the following parameters were evaluated: skin, fur, mucous membranes, respiration, nervous system function (including tremors and convulsions), animal behavior, moribundity, mortality, and the availability of feed and water. Because the ears of the CBA/J mice are brown, erythema evaluations of the ear surfaces were not assessed.

CALCULATION AND STATISTICS

1. The % ear swelling was calculated for each ear using the following equation:

$$\% \text{ Ear swelling} = \frac{B - A}{A} \times 100 \text{ where:}$$

A = mean of pretreatment measurement (mm x 10⁻²)

B = mean of post treatment measurement (mm x 10⁻²)

2. The Stimulation Index (SI) was calculated for each mouse using the following equation:

$$SI = \frac{\text{Disintegration per minute (dpm) of individual mouse}}{\text{Average dpm of the VH control mice}}$$

Descriptive statistics only (means and standard deviations) are reported for ear irritation data. Body weights (absolute and gains) and lymphocyte proliferation data were first evaluated by Bartlett's test ($\alpha = 0.01$; Winer, 1971) to determine equality of variances. Based upon the outcome of Bartlett's test, either a parametric (Steele and Torrie, 1960) or non-parametric (Hollander and Wolfe, 1973) analysis of variance (ANOVA) was performed.

If the ANOVA was significant at $\alpha = 0.05$, it was followed respectively by Dunnett's test ($\alpha = 0.05$; Winer, 1971) or the Wilcoxon Rank-Sum test ($\alpha = 0.05$; Hollander and Wolfe, 1973) with a Bonferroni correction (Miller, 1966) for multiple comparisons to the control. Statistical outliers were identified by a sequential test ($\alpha = 0.02$; Grubbs, 1969). The final interpretation of the biological significance of the responses was based on both statistical outcome and scientific judgement.

RESULTS AND DISCUSSION

Body weight data were unremarkable (Appendices IA and 1B). Topical applications of HBCD elicited slight ear swelling at doses of 10%, 25%, and 50% w/v during the screening phase of the study (Table 1). Based upon these data, HBCD was subsequently tested in the LLNA at 2%, 20%, and 50% w/v.

Lymphocyte proliferation by DMF, vehicle treated mice (2,015 dpm) was higher than historical laboratory values (Table 3) commonly observed using acetone and olive oil as a vehicle. This is not inconsistent with that reported in the literature for this vehicle. One mouse in particular demonstrated 3481 dpm in its draining lymph nodes and was confirmed to be a statistical outlier. Exclusion of this vehicle treated animal still resulted in a control value of 1,722 dpm and did not ultimately alter the conclusions of this study.

HCA administrations (30% v/v) elicited proliferation that was 3-fold greater than that of vehicle controls. Exclusion of the statistical outlier in the vehicle group increased the HCA SI value to above 3.5. While this SI value for HCA is slightly lower than reported values when diluted in acetone and olive oil, HCA was still positive according to guideline criteria and demonstrated ^3H -thymidine uptake (5,970 dpm) that was

statistically higher than that of vehicle controls (2,015 dpm). HCA is a useful positive control for the LLNA as it is a moderate contact sensitizer and can serve as a better indication of the assay's sensitivity compared with extreme sensitizers such as DNCB.

HBCD did not demonstrate results that were consistent with dermal sensitization (Table 2). SI values were consistently around 1.0 at all doses tested.

CONCLUSIONS

HBCD did not elicit lymph node proliferation when tested as high as 50% w/v in the mouse LLNA. HBCD has also been tested in the guinea pig using a Magnusson and Kligman (M&K) Maximization test, and in a human patch test with fabric treated with HBCD. The human patch test did not elicit skin sensitization (McDonnell 1972). In the M&K test, a lack of dermal sensitization was reported by Wenk (1996), whereas Momma *et al.* (1993) and Nakamura *et al.* (1994) reported positive results. Specifics of the test material used by Momma and Nakamura were not described in detail and may partially explain differing results. Though both the guinea pig M&K and the LLNA are both considered appropriate for evaluating contact sensitization potential, the LLNA has the advantage of a quantitative, dose-response endpoint for sensitization potential. These results suggest that HBCD does not possess contact sensitization potential.

ACKNOWLEDGEMENTS

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B. K. Hall	Document Management
J. W. Lacher	Clinical Veterinarian
J. Murray	Veterinarian Assistant
J. M. Rase	Technical Assistance
J. L. Fairchild	Animal Welfare
D. W. Louch	Animal Welfare

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HEXABROMOCYCLODODECANE: CONTACT SENSITIZATION POTENTIAL VIA THE LOCAL
LYMPH NODE ASSAY IN CBA/J MICE

Table 1. Percent Ear Swelling (Screen)

Treatment	Percent Ear Swelling (mean)
Vehicle (DMF)	1.0
1% HBCD	2.0
5% HBCD	6.2
10% HBCD	19.8
25% HBCD	26.5
50% HBCD	19.8

Mice (n=1/group) were treated topically with vehicle or HBCD preparation for two consecutive days. Ear thickness was measured using a digital micrometer. Values represent mean for each mouse (n=2 ears/dose). DMF = N,N-Dimethylformamide
HBCD = hexabromocyclododecane

HEXABROMOCYCLODODECANE: CONTACT SENSITIZATION POTENTIAL VIA THE LOCAL
LYMPH NODE ASSAY IN CBA/J MICE

Table 2. Summary of Lymph Node Proliferation Data

Treatment Group	DPM (mean \pm SD)	SI (mean \pm SD)
Vehicle (DMF)	2015 \pm 749	1.0 \pm 0.4
HCA (30% v/v)	5970 \pm 1909*	3.0 \pm 0.9
2% HBCD	1744 \pm 181	0.9 \pm 0.1
20% HBCD	1670 \pm 155	0.8 \pm 0.1
50% HBCD	1946 \pm 664	1.0 \pm 0.3

Mice (n=6/group) were treated topically with vehicle or HBCD preparation for 3 consecutive days. Mice were injected intravenously (tail) with ^3H -thymidine and draining auricular lymph nodes were evaluated for ^3H -thymidine incorporation into proliferating lymphocytes. Values represent mean and standard deviations (S.D.). * represents statistical difference from control mean at $p \leq 0.05$ using Dunnett's T-test.

DMF = N,N-Dimethylformamide; HBCD = hexabromocyclododecane;

HCA = α hexyl cinnamic aldehyde; DPM = disintegrations per minute; SI = stimulation index

HEXABROMOCYCLODODECANE: CONTACT SENSITIZATION POTENTIAL VIA THE LOCAL
LYMPH NODE ASSAY IN CBA/J MICETable 3. Historical Values of Vehicle (4:1 Acetone:Olive Oil) Responses from LLNA Studies
Conducted in Toxicology & Environmental Research and Consulting

DATE	ABSOLUTE DPM
May 2003	681 \pm 252
May 2003	438 \pm 121
May 2003	613 \pm 20
April 2003	539 \pm 147
September 2002	561 \pm 35
August 2002	353 \pm 55
July 2002	276 \pm 58
July 2002	561 \pm 35
May 2002	490 \pm 139
May 2002	237 \pm 30
April 2002	392 \pm 100
January 2002	548 \pm 116
October 2001	345 \pm 70
September 2001	739 \pm 151
September 2001	240 \pm 36
July 2001	724 \pm 188
January 2001	255 \pm 22
January 2001	385 \pm 16
Average	465 \pm 165

HEXABROMOCYCLODECANE: CONTACT SENSITIZATION POTENTIAL VIA THE LOCAL LYMPH NODE ASSAY IN CBA/J MICE

Appendix IA. Individual Body Weights (g) of Mice Treated with Vehicle and Positive Control (HCA)

DOSE %	ANIMAL NUMBER	DAYS ON TEST		
		9	14	GAIN
0	459	21.0	22.3	1.3
	460	21.4	22.9	1.5
	461	21.4	23.0	1.6
	462	23.0	23.6	0.6
	463	21.1	22.2	1.1
	464	23.4	23.9	0.5
	MEAN	21.9	23.0	1.1
	S.D.	1.0	0.7	0.5
	N=	6	6	6
	30	465	22.1	22.8
466		23.0	23.5	0.5
467		22.9	23.6	0.7
468		19.8	21.0	1.2
469		22.2	22.0	-0.2
470		23.4	24.0	0.6
MEAN		22.2	22.8	0.6
S.D.		1.3	1.1	0.5
N=		6	6	6

0 = vehicle (DMF N,N-Dimethylformamide)
30 = HCA = α -hexyl cinnamic aldehyde tested at 30% v/v
g = grams; S.D. = standard deviation; N = number of animals in group

HEXABROMOCYCLODODECANE: CONTACT SENSITIZATION POTENTIAL VIA THE LOCAL LYMPH NODE ASSAY IN CBA/J MICE

Appendix IB. Individual Body Weights (g) of Mice Treated with HBCD

DOSE HBCD	ANIMAL NUMBER	DAYS ON TEST		
		9	14	GAIN
=====				
2	471	20.7	22.6	2.5
	472	22.5	23.4	2.7
	473	20.8	22.4	3.1
	474	21.9	22.5	2.2
	475	22.2	22.7	0.3#
	476	22.9	24.8	2.8
	MEAN	21.8	23.1	2.3
S.D.	0.9	0.9	1.0	
N=	6	6	6	
=====				
20	477	22.2	23.2	2.8
	478	21.0	22.9	3.3
	479	21.2	23.2	3.0
	480	23.2	24.7	2.8
	481	23.7	23.7	2.6
	482	24.5	25.0	2.6
	MEAN	22.6	23.8	2.9
S.D.	1.4	0.9	0.3	
N=	6	6	6	
=====				
50	483	22.5	24.5	3.1
	484	20.1	22.0	1.6
	485	23.0	23.3	2.6
	486	20.6	21.8	2.0
	487	20.8	21.2	1.4
	488	25.1	25.1	1.9
	MEAN	22.0	23.0	2.1
S.D.	1.9	1.6	0.6	
N=	6	6	6	
=====				

Statistical Outliers Included

HBCD=hexabromocyclododecane; g = grams; S.D. = standard deviation; N = number of animals in group

HEXABROMOCYCLODECANES: CONTACT SENSITIZATION POTENTIAL VIA THE LOCAL LYMPH NODE ASSAY (INCLUDING A PRIMARY IRRITANCY SCREEN) USING CBA/J MICE

Appendix IIA. Individual DPM and SI of Mice Treated with Vehicle and Positive Control (HCA)

DOSE %HCA	ANIMAL NUMBER	DPM	SI
=====			
0	459	3481#	1.7#
	460	1757	0.9
	461	1459	0.7
	462	1872	0.9
	463	1504	0.7
	464	2019	1.0
	MEAN	2015	1.0
	S.D.	749	0.4
	N=	6	6
=====			
30	465	9133	4.5
	466	4884	2.4
	467	5937	2.9
	468	5391	2.7
	469	6915	3.4
	470	3559	1.8
	MEAN	5970	3.0
	S.D.	1909	0.9
	N=	6	6
=====			
# Statistical Outliers Included.			
0=vehicle (DMF N,N-Dimethylformamide)			
HCA = 30=30% α hexyl cinnamic aldehyde			
DPM=disintegrations per minute			
SI=stimulation index;			
S. D. = standard deviation; N = number of animals in group			

HEXABROMOCYCLODODECANE: CONTACT SENSITIZATION POTENTIAL VIA THE LOCAL LYMPH NODE ASSAY (INCLUDING A PRIMARY IRRITANCY SCREEN) USING CBA/J MICE

Appendix IIB. Individual DPM and SI of Mice Treated with HBBCD

DOSE %HBBCD	ANIMAL NUMBER	DPM	SI
=====			
2	471	1617	0.8
	472	1902	0.9
	473	1909	0.9
	474	1504	0.7
	475	1629	0.8
	476	1901	0.9
	MEAN	1744	0.8
	S.D.	181	0.1
	N=	6	6
=====			
20	477	1688	0.8
	478	1650	0.8
	479	1637	0.8
	480	1491	0.7
	481	1954	1.0
	482	1599	0.8
	MEAN	1670	0.8
	S.D.	155	0.1
	N=	6	6
=====			
50	483	1546	0.8
	484	1386	0.7
	485	1784	0.9
	486	3238#	1.6#
	487	1762	0.9
	488	1960	1.0
	MEAN	1946	1.0
	S.D.	664	0.3
	N=	6	6
=====			

Statistical Outliers Included

DPM=disintegrations per minute

SI=stimulation index

HBBCD=hexabromocyclododecane; S.D. = standard deviation; N = number of animals in group

STUDY TITLE

EVALUATION OF AEROBIC AND ANAEROBIC TRANSFORMATION OF
HEXABROMOCYCLODODECANE IN SOIL

Author(s)

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Study Completion Date

05 March 2003

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The Dow Chemical Company
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Laboratory Project Study ID

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COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

Compound: HEXABROMOCYCLODODECANE

Title: EVALUATION OF AEROBIC AND ANAEROBIC TRANSFORMATION
OF HEXABROMOCYCLODODECANE IN SOIL

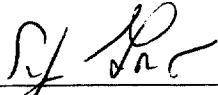
All phases of this study were conducted in compliance with the following Good
Laboratory Practice Standards:

US Environmental Protection Agency-TSCA GLPS

Title 40 CFR, Part 792-Toxic Substances Control Act (TSCA); Good Laboratory Practice
Standards, Final Rule


OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring,
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QUALITY ASSURANCE STATEMENT

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This study was examined for conformance with Good Laboratory Practices as published by the USEPA (TSCA), EC and the OECD. The dates of Quality Assurance activities on this study are listed below.

Study Initiation Date: 20 June 2002

<u>TYPE OF AUDIT:</u>	<u>DATE OF AUDIT:</u>	<u>DATE FINDINGS REPORTED TO STUDY DIRECTOR/MANAGEMENT:</u>
Final protocol	27 June 2002	27 June 2002
Study conduct	17 July 2002	17 July 2002
Protocol, data, and draft report	26 February 2003	28 February 2003
Final report	The date of the signature below is the date of the final report audit.	

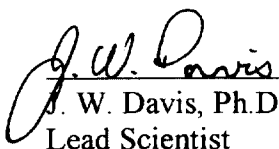
The final report accurately reflects the raw data of the study.

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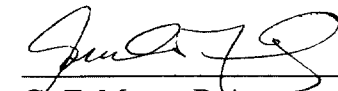
SIGNATURE PAGE

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SUMMARY

The transformation of hexabromocyclododecane (HBCD) was determined in aerobic and anaerobic soils based on the Organisation for Economic Co-Operation and Development (OECD) Test Guideline 307 "Aerobic and Anaerobic Transformation in Soil." Soil microcosms were prepared by adding a sandy loam surface soil to serum bottles sealed with Teflon[®] coated septa. Aerobic microcosms were prepared by adjusting the soil moisture to 20% (by weight) and periodically exchanging the headspace of the microcosms with ambient air to replenish oxygen. The microcosms were pre-incubated at 20 ± 1 °C for 35 days. Anaerobic microcosms were prepared in an anaerobic atmosphere (70% N₂, 28% CO₂, and 2% H₂) by flooding the soil with water and pre-incubating the microcosms at 23 ± 1 °C for 43 days to allow low redox (e.g., methanogenic) conditions to develop. HBCD was then added to microcosms at a nominal concentration of 25 ng/g (soil dry weight), together with activated sludge (5 mg/g, dry weight basis) from a municipal wastewater treatment plant to simulate sludge land treatment applications. Biologically inhibited (abiotic) controls were prepared by steam sterilization prior to the addition of HBCD. Microcosms were incubated in the dark at 20 ± 1 °C for 119 days. The concentration of HBCD in the microcosms was determined at selected time intervals utilizing high performance liquid chromatography-mass spectrometry (LC-MS). Aerobic microcosms were analyzed on days 0, 1, 7, 21, 48, 65, and 119, while anaerobic microcosms were analyzed on days 0, 1, 7, 21, 56, 91, and 119.

HBCD concentrations decreased over time in both the aerobic and anaerobic soils. HBCD concentrations decreased 75% over 119 days in the viable aerobic soil microcosms, compared to a 3% decrease in the abiotic controls, indicating that biological processes were responsible for most of the losses observed. Under anaerobic conditions, HBCD concentrations decreased 92% over 21 days in the viable microcosms compared to a less than 1% decrease in the abiotic controls. Pseudo-first order kinetics rate constants for the biotransformation of HBCD were determined by subtracting the abiotic rate constant from the viable rate constant. Biotransformation half-lives for HBCD were determined to be 63 and 6.9 days in the aerobic and anaerobic soils, respectively.

Brominated degradation products were not detected in the soil or in the headspace of the microcosms. Based upon these results HBCD can not be considered “persistent in the environment” since the half-lives in soil are clearly below the criteria for persistence (in soil) as specified in various international protocols (UNECE 1966, UNEP 2001).

INTRODUCTION

Hexabromocyclododecane (HBCD) is used as a flame retardant in several applications, including expanded and extruded polystyrene. HBCD has the potential for release into the environment from production processes, and from the processing and disposal of fabricated products containing the compound.

Biodegradation is a major process for removing chemicals from the environment. Aerobic biodegradation processes can predominate in surface waters, surface soils, and the aeration basins of wastewater treatment plants (WWTPs). Anaerobic processes, in contrast, can occur in aquatic sediments, groundwater, and in anaerobic digestion units of WWTPs. Little information is available concerning the aerobic and anaerobic biodegradability of HBCD. The compound exhibited no degradation in the OECD 301D Closed Bottle Test [1]. However, these results may have minimal environmental consequences since the test concentration of HBCD exceeded the reported water solubility of 3.4 µg/L by greater than 2000-fold. The lack of measurable biodegradation in this test was due, in part, to the limited availability of the test material to the microorganisms as well as the low amount of biomass contained in the inoculum.

The objective of this study was to assess the lifetime of HBCD at realistic environmental concentrations under both aerobic and anaerobic conditions in soil. The environmental behavior of HBCD is governed by how it partitions between soil, water and air. Partitioning and transport of HBCD is dependent upon its intrinsic physical-chemical properties including a low vapor pressure (6.3×10^{-5} Pa), low aqueous solubility (3.4 µg/L), high K_{ow} (Log 5.62) and high soil organic carbon sorption coefficient (Log K_{oc} = 4.66) [1]. Based upon these properties there is a high potential for this material to absorb to soil and sediments. In order to predict the environmental lifetime of a chemical it is important to demonstrate degradation in the environmental compartments

where the compound is likely to reside. Since soil and sediments are likely sinks for HBCD it is critical to quantitate the degradation processes in these compartments to better predict the environmental lifetime of HBCD.

The experimental procedure was based on the Organisation for Economic Co-Operation and Development (OECD) Guideline 307 "Aerobic and Anaerobic Transformation in Soil" [2]. Aerobic and anaerobic biodegradation studies were conducted in laboratory batch microcosms prepared with a surface soil from Northwood, North Dakota. The concentration of HBCD in the soil was determined at selected time intervals using high performance liquid chromatography/mass spectrometry (LC/MS). The analysis allowed the primary biodegradation of HBCD to be followed, and transformation rates to be determined.

MATERIALS

Test Material

A 25-gram sample of HBCD was obtained from Wildlife International, Ltd., Easton, Maryland on January 8, 2002. The sample was identified as a "Representative Sample of Composite of HBCD – WIL# 5850," with a lot number MC258500102. The sample was a composite of test materials supplied by Albemarle Corporation, Great Lakes Chemical Corporation, and Eurobom B.V. The identity and composition of the sample was characterized by Albemarle Corporation [3]. The identity of the compound was confirmed by Fourier Transform Infrared Spectroscopy. The composition of the test sample, based on the average of triplicate analyses of six separate sub-samples collected from different locations in the bulk sample, was 5.8% alpha isomer, 19.3% beta isomer, and 74.9% gamma isomer. The molecular formula for HBCD is $C_{12}H_{18}Br_6$ ($M_w = 641$).

Hexanes (Optima grade) solvent for sample extractions and anhydrous sodium sulfate (Certified ACS) used to dry sediment samples were obtained from Fisher Scientific, Pittsburgh, Pennsylvania. Laboratory water was purified in a MilliQ[®] Water Purification System (Millipore Corporation, New Bedford, Massachusetts).

Soil Sample

Agvise Laboratories (Northwood, North Dakota) collected a sandy loam soil (USDA classification) on April 16, 2002. The top six inches of soil was collected with a hand shovel. The soil sample was dispensed into seven metal cans, packed with blue ice, and shipped via overnight air express to The Dow Chemical Company in Midland, Michigan. The soil sample was passed through a 2-mm sieve to remove stones, plant matter and to improve sample homogeneity and stored at approximately 4 °C until used. Agvise Laboratories characterized a sub-sample of the collected soil for texture classification, percent organic carbon, pH, cation exchange capacity, bulk density, moisture, major cations, and microbial biomass.

Activated Sludge Sample

Activated sludge was obtained from the Midland Municipal Wastewater Treatment Plant (Midland, Michigan) on July 2, 2002. The activated sludge sample was washed twice with tap water by allowing the sludge to settle and replacing the supernatant liquid with tap water. The sludge sample was concentrated by centrifugation at approximately 3,500 rpm for 20 minutes in a Sorvall Centrifuge. The sludge was removed and the solids content was determined to be 15% using a standard procedure [4]. The sludge was refrigerated at approximately 4 °C until used.

EXPERIMENTAL METHODS

Test Procedure – Aerobic Soil Reaction Mixtures

A series of soil microcosms were prepared by adding portions of soil (50-g dry weight) to 250-mL serum bottles. The moisture content of the soil was determined using a standard procedure [5]. The serum bottles were described as I-Chem 200- and 300-series (I-Chem Company, New Castle, Delaware) that were cleaned and certified by the manufacturer to meet U.S. EPA analyte specifications. The moisture content of the soil was adjusted from approximately 16% to 20% by weight by the addition of 3 mL of laboratory water to each microcosm. This moisture content was within the range of 40 to 60 percent of the water holding capacity as specified by the test guideline [2]. The serum bottles were

sealed with Teflon[®]-coated rubber septa contained in screw-on caps. The microcosms were stored at 20 ± 1 °C for 35 days (stabilization period) to re-establish equilibrium of microbial metabolism following the change from sampling and storage conditions to test incubation conditions [2]. The headspace of the viable microcosms was exchanged with ambient air on a weekly basis during the stabilization period to replenish oxygen consumed by biological activity.

Following the stabilization period, the screw-on caps were removed and the headspace gases of the microcosms were allowed to exchange with ambient air. A 1.7 gram portion of activated sludge (15% solids) was added to each microcosm resulting in a final sludge concentration of 5 mg of mixed liquor suspended solids per gram of dry soil. This level of sludge addition is consistent with application rates for sewage sludge/farming application [6]. HBCD was added to replicate test mixtures at an approximate nominal concentration of 25 ng/g (based on soil dry weight) as described in Table 1. This initial HBCD concentration was chosen to ensure that any degradation/removal of HBCD in the soil could be followed through at least two half-lives. A 5-μL volume of an acetone stock solution containing 250 mg/L HBCD was added to each microcosm. Background microcosms, included in the study design to identify possible analytical interferences, received only 5-μL of acetone. The soil mixtures were mixed with a spatula to distribute the HBCD and sludge throughout the soil. The microcosms were left unsealed for approximately one hour to allow the acetone to evaporate. Biologically inhibited controls were prepared by steam sterilizing (121 °C; 15 psi; 60 minutes) the soil on three separate days prior to the addition of the test compound. The activated sludge added to the biologically inhibited controls received one steam sterilization treatment.

Test Procedure – Anaerobic Soil Reaction Mixtures

Microcosms for the anaerobic transformation experiment were prepared in an anaerobic atmosphere and covered with water to allow anaerobic conditions to develop. A series of soil microcosms were prepared by adding portions of soil (50-g dry weight) to 250-mL serum bottles. The microcosms were transferred to an anaerobic glove box with an anaerobic atmosphere (~70% (vol) N₂, ~28% CO₂, and ~2% H₂). Twenty-mL portions of

steam sterilized tap water, which was previously sparged for 10 minutes with zero-grade nitrogen, were added to each of the microcosms to cover the soil. The microcosms were sealed and incubated in an anaerobic atmosphere at 23 ± 1 °C for 43 days to allow methanogenic conditions to develop. A subset of anaerobic microcosms were analyzed for pH and redox potential once per week. Resazurin, a redox dye, was added to this subset of microcosms at 1 mg/kg to indicate when the redox potential dropped below -110 mV [7]. The headspace of selected microcosms was monitored for methane gas to confirm methanogenic conditions. Methane gas formation was monitored by transferring 1 mL of headspace gas from selected microcosms to 10-mL serum bottles capped with septa. One mL of the serum bottle gas was analyzed by GC-FID to determine the methane concentration.

Following the acclimation period (defined by the presence of methane), the test microcosms were spiked with HBCD according to the experimental design described in Table 1. HBCD and activated sludge (5 mg of mixed liquor suspended solids per gram of dry soil) were added to the microcosms in the same manner as described above for the aerobic microcosms. Biologically inhibited (abiotic) controls were prepared in the same manner as the test microcosms, except that the microcosms were steam sterilized on three separate days prior to the addition of the test material. The activated sludge that was added to the abiotic controls was also steam sterilized. The microcosms were amended with sludge and HBCD in an anaerobic atmosphere to ensure that anaerobic conditions were maintained.

Microcosm Incubation

All microcosms were incubated in the dark at 20 ± 1 °C. Headspace gases in the viable aerobic microcosms were exchanged with laboratory air after 14, 80, and 101 days of incubation to ensure sufficient concentrations of oxygen in the headspace. The oxygen concentrations in the headspace of the viable microcosms was routinely measured before sacrificing microcosms for analysis to confirm that aerobic conditions were maintained. Oxygen analyses were conducted using a fiber optic probe that was protected in a #18

gauge stainless steel needle that was inserted through the septum into the serum bottle headspace.

Microcosms were prepared for analysis as follows: The soil in the microcosms was dried with anhydrous sodium sulfate (25 g for aerobic soils and 75 g for anaerobic soils) and mixed with 100 mL hexanes on a reciprocating shaker for approximately one day. Matrix spikes were used to determine the recovery of HBCD from the soil matrix. Matrix spikes were prepared adding 5 μ L of an HBCD stock solution (250 mg/L in acetone) to background microcosms prior to the addition of the extraction solvent. At the beginning of the study, single matrix spikes were prepared. As the study progressed, duplicate matrix spikes were prepared to improve the reproducibility of the HBCD assay. Background microcosms (no HBCD added) were extracted and analyzed for potential background interferences on selected days. Method spikes were used to monitor the extraction efficiency of HBCD in the assay. Method spikes were prepared by adding 5 μ L of the HBCD stock solution to 100 mL of laboratory water and extracting for approximately one day with 30-mL hexanes. Details of the sample preparations are described in Appendix A.

At the conclusion of the study, bromide levels were measured in selected microcosms to determine if bromide was released as a result of HBCD degradation. Water (50 mL) was added, the microcosms were vigorously mixed for several minutes, and then allowed to settle overnight. The purpose of the mixing was to uniformly distribute any bromide ions in the aqueous phase. The aqueous layer was centrifuged to remove solids and the clarified supernatant liquid layer was analyzed for bromide by ion chromatography.

At the conclusion of the study, the headspace of selected background and HBCD amended microcosms was analyzed for the presence of volatile brominated degradation products. An adsorbent carbon tube (SKC Anasorb CSC, Coconut Shell Charcoal) was connected between a 60-mL plastic syringe and a syringe needle that was inserted through the septum of the microcosm. A second needle was inserted through the septum with the tip positioned above the soil. Flushing the headspace of the microcosm through the carbon adsorbent was accomplished by drawing approximately 300 mL of ambient air

(5 x 60-mL portions) through the microcosm headspace. The carbon adsorbent was extracted with a 1.3 mL of acetonitrile for at least 16 hours, mixed with 0.2 mL of acidified water, and analyzed for brominated degradation products by LC-mass spectrometry and GC-mass spectrometry.

For the aerobic soil microcosms, single background and duplicate test (i.e., HBCD amended) microcosms (viable and abiotic) were analyzed. The viable test microcosms were opened after 80 days of incubation to replenish depleted oxygen levels by headspace gas exchange with ambient air. The microcosms were sealed for the remainder of the study (39 days of additional incubation) to allow possible volatile degradation products to accumulate in the microcosm headspace. The abiotic microcosms were not opened during the study. For the anaerobic microcosms, the headspace of five microcosms (single viable background and duplicate test; single abiotic background and test) that were sealed for the entire study were sampled and analyzed.

Analytical Methods

A high performance liquid chromatography/mass spectrometry (LC/MS) method was used to measure HBCD concentrations in this study. This assay was derived from a method previously used for the analysis of HBCD in water and fish tissues [8,9]. Extensive method development was required to identify sample extraction and instrument parameters that would provide a sensitive, reproducible assay for the environmentally realistic concentrations of HBCD used in this study. Initial method development was performed on a PE Sciex API 2000 LC/MS/MS using atmospheric pressure chemical ionization (APCI). Hexanes, toluene, isooctane, and methylene chloride gave comparable extraction efficiencies for HBCD in environmental matrices. Hexanes was chosen as the extraction solvent for the HBCD assay because its low density (i.e., floats on water) and volatility were ideally suited to recover and concentrate HBCD residues from the microcosms used in this study. Due to instrument problems, the PE Sciex API 2000 LC/MS/MS system was replaced with an Agilent Technologies LC-MS with atmospheric pressure photoionization (APPI) prior to the start of the HBCD transformation studies.

Sample work-up prior to LC-MS analysis consisted of the following: Hexanes extracts of the soil samples were washed to remove salts, evaporated to dryness, and reconstituted in acetonitrile/water (85/15) containing 0.1% HCl. The samples were analyzed by LC-MS with APPI. Instrument conditions are included in Appendix B. The transformation of HBCD was followed by monitoring the HBCD molecular ion ($m/z = 641$ amu) and its bromine fragment ion ($m/z = 79$ amu). Representative spectra for a method standard are shown in Figure 1. By monitoring the bromine fragment ion, the analysis provided the potential to detect brominated degradation products. Concentrations of HBCD were quantified based on the HBCD molecular ion ($m/z = 641$ amu). The three isomers present in the test material (alpha, beta, and gamma) could be resolved in the LC-MS assay. However, only the major gamma isomer (75% of HBCD) was used to quantify HBCD concentrations in the microcosms (the alpha and beta isomers would have been detected had they been present at levels similar to the starting gamma concentration). Calibration curves were generated at the beginning and end of each sample set to confirm proper operation of the instrumentation and linearity of detector response. To compensate for any change in response of the HPLC-MS, a reference standard was repeatedly analyzed throughout the sample set (*e.g.*, every fourth or fifth analysis). Response factors were determined for the reference standard and an average response factor was used to calculate HBCD concentrations for samples bracketed by the reference standards. Measured HBCD concentrations were corrected for recovery based on matrix spikes prepared for each sample set. Method spikes (water spiked with HBCD) were included in each sample set to confirm the reliability of the extraction procedure. The average recovery of HBCD in the method spikes prepared over a 127 day period was $93 \pm 13\%$ (Appendix C). HBCD was stable in stock solutions prepared in acetone that were used to dose the test microcosms and prepare calibration standards. The HBCD stock solution used to spike the test microcosms was re-assayed with a fresh stock solution prepared 106 days later. The results showed 109% of the original concentration of HBCD present, confirming the stability of the HBCD stock solution.

Though authentic standards of possible HBCD degradation products were not commercially available, four brominated aliphatic compounds were evaluated in the

LC-MS assay to assess the capability to detect brominated compounds. The four compounds, hexabromocyclohexane, 1,2-dibromocyclooctane, trans 1,2-dibromocyclohexane, and 1,10-dibromodecane, each showed a response in the assay at 79 m/z. Thus, the LC-MS assay was capable of detecting a range of brominated compounds indicating that this analysis had the potential for detecting brominated products which could typically be produced from HBCD degradation.

The acetonitrile extracts from the carbon adsorbent tubes used to collect possible volatile brominated degradation products of HBCD from the microcosm headspace were analyzed by LC-MS and GC-MS without further preparation. The bromine fragment ion ($m/z = 79$ amu) was monitored to detect possible brominated degradation products. Instrument conditions for the GC-MS assay are included in Appendix D.

Bromide concentrations in selected aqueous samples were determined using ion chromatography. Instrument conditions are listed in Appendix E.

Methane concentrations in selected anaerobic microcosms were measured by GC with flame ionization detection (FID). Instrument conditions are listed in Appendix F. Methane gas standards were prepared by diluting a gas standard containing 10% (mole %) methane, ethane, and ethylene (BOC Gases, Port Allen, Louisiana) with air in septum-capped headspace vials. The methane concentration in the headspace of the selected microcosms was determined using an external standard calculation.

Oxygen concentration in the headspace of the microcosms was measured using an S2000 Fiber Optic Spectrometer (Ocean Optics, Inc., Dunedin, Florida). The fiber optic sensor was calibrated daily using ambient air (20.95% oxygen) and argon gas (0% oxygen) contained in a sealed serum bottle.

The pH of aqueous samples was determined using a standard procedure [10]. An Orion Sure-Flow Ross Semi-Micro Electrode was connected to an Orion model 920-pH meter. The pH probe was calibrated with standard buffered solutions at pH 4, 7, and 10 (Fisher Scientific International, Hampton, New Hampshire).

The oxidation-reduction potential (Eh) of the aqueous layer of selected anaerobic microcosms was measured using an Accumet probe connected to an Orion model 920 meter. ZoBell's solution ($3 \times 10^{-3}M$ potassium ferrocyanide and $3 \times 10^{-3}M$ potassium ferricyanide in 0.01M KCl) was prepared to calibrate the probe. The Eh value was determined by correcting the E_{observed} value. The correction was based on the measured Eh value for the Zobell's solution and temperature [11].

Temperature measurements in the anaerobic chambers were made with ASTM 63 thermometers [12]. Temperature measurements in the incubators were made using an automated laboratory system [13].

RESULTS

Soil Characterization

The soil was classified as a sandy loam (United States Department of Agriculture classification system) with 64% sand; 20% silt, and 16% clay. The pH was 6.4, cation exchange capacity 19.2 meq/100 g, bulk density 1.11 g/cc, and moisture at 1/3 bar of 23.9 percent. The percent organic carbon by combustion was 1.8%. Major cations included 2240 ppm calcium; 529 ppm magnesium; 47 ppm sodium; 201 ppm potassium. Microbial biomass content was determined to be 274 µg/g dry weight soil using an extraction method [14].

Stabilization of Soil Microcosms

The soil microcosms were incubated for 35 (aerobic) to 43 days (anaerobic) at approximately 20 °C prior to the addition of HBCD. This stabilization period was recommended by the test guideline to reestablish equilibrium of microbial metabolism following the change from sampling and storage conditions to incubation conditions [2]. The total storage and stabilization time of the soils did not exceed three months, consistent with the guideline recommendations. On the day prior to the end of the stabilization period (42 days), oxygen was not detected in the headspace of five selected anaerobic microcosms. These results confirmed that oxygen was absent from the microcosms, thereby supporting the formation of low redox conditions in the soil.

Methane concentrations in the headspace of selected anaerobic microcosms were monitored during the stabilization phase to further verify that a low redox potential was achieved in the anaerobic soils. No detectable methane was measured in the headspace after 9, 16, 24, and 29 days of incubation. After 42 days of incubation, prior to the addition of HBCD, methane was present at 0.09 ± 0.07 % ($n = 4$) in the headspace of the anaerobic microcosms. These results further indicate a low redox potential and that anaerobic conditions were established in the microcosms during the stabilization phase.

The pH and redox potential for selected anaerobic soil microcosms were regularly monitored during the stabilization phase. Measurements of pH and redox potential were

made in the water layer immediately above the soil (Table 2). The pH of the water remained between pH 6.1 and 6.5 during the stabilization phase, and increased to pH 6.8 at the conclusion of the study (120 days). Redox potentials in the water remained in the range of 132 to 291 mV during the stabilization phase. The resazurin dye, added as a visual indicator of redox potential, was colorless, indicating a low redox potential. The reason for the positive values measured with the redox probe is not known. However, other researchers have encountered problems with accurately measuring redox potentials in environmental systems since redox couples are often not in equilibrium [15].

Lifetime of HBCD in Soil

HBCD concentrations decreased over time in viable microcosms under both aerobic and anaerobic conditions. In the viable aerobic soil microcosms, HBCD concentrations decreased from 15.9 to 4.0 ng/g (based on dry weight of soil) over 119 days, a decrease of 75% (Table 4 and Figure 2). HBCD concentrations decreased 3% in the corresponding abiotic controls (18.0 to 17.4 ng/g) over the same time period. The measured day 0 concentrations of HBCD in the viable and abiotic control microcosms were 64 and 72% of the nominal concentration of HBCD (25 ng/g), respectively.

Figures 3 through 6 include LC-MS chromatograms that illustrate the loss of HBCD in the aerobic soil. The upper chromatogram in each figure shows the molecular ion of HBCD (641 m/z) while the lower chromatogram shows the bromine fragment (79 m/z). Note the decrease in both peaks over time for day 0 (Figure 3), day 65 (Figure 4), and day 119 (Figure 5) viable microcosms. In contrast, HBCD remains in the abiotic control on day 119 (Figure 6). Note that the alpha and beta isomers of HBCD were not detected after day 0. These results indicate that the major gamma isomer of HBCD did not convert to the alpha and beta isomers in this test system. Also, note that there were no detectable bromine fragment ions peaks at retention times other than the HBCD retention time (i.e., no brominated degradation products were detected).

In the viable anaerobic soil microcosms, HBCD concentrations decreased from 11.0 to 0.9 ng/g within 21 days, a decrease of 92% (Table 5 and Figure 7). No decrease of

HBCD was observed in the abiotic controls over the same time period. HBCD concentrations decreased 60% in the abiotic controls after 119 days (17.2 to 6.9 ng/g).

Figure 8 (day 0) and Figure 9 (day 119) illustrate the loss of HBCD in the viable anaerobic soil microcosms. No conversion of the major gamma isomer to the minor isomers was observed, and no brominated degradation products were detected in the study.

Kinetics of HBCD Removal

Degradation rates of chemicals are routinely approximated by using kinetics that are first order with respect to substrate concentration. This approach is commonly used when chemicals are present at low concentrations in the environment (low $\mu\text{g/L}$ or $\mu\text{g/kg}$). Pseudo-first order kinetics can be assumed when there are negligible changes in biomass levels that occur as a result of growth [16]. In this study pseudo-first order rate constants for HBCD removal in the soil microcosms were determined from plots of the natural logarithm of the HBCD concentration versus time (Appendix G). For the purposes of this calculation, data points collected between day 0 and day 21 (92% disappearance of HBCD) were used to determine the transformation rate constant for the viable anaerobic microcosms. Results of the kinetic analysis are summarized in Table 6. Pseudo-first order biotransformation rate constants were determined by subtracting the abiotic rate constant (abiotic microcosms) from the viable rate constant. Based on these rate constants, the biotransformation half-life for HBCD was 63 days in the aerobic soil microcosms and 6.9 days in the anaerobic soil microcosms. Note that, when compared to the rates observed in abiotic controls, the biologically mediated transformation rates were 12-fold faster in the anaerobic soils and up to 110-fold faster in the aerobic soils.

HBCD Degradation Products

HBCD degradation products were not detected in extracts of the soils. The LC-MS assay routinely monitored the HBCD molecular ion at 641 amu and the bromine 79 amu fragment ion in each sample analyzed. Degradation products containing bromine would be expected to produce a bromine fragment ion and thus be detected in the assay.

At the conclusion of the study, the headspace of selected microcosms was passed through carbon adsorbent to collect possible volatile brominated degradation products. Organic extracts of the carbon tubes were analyzed by LC-MS and GC-MS. No brominated compounds were detected in the assays in which the bromine fragment ion ($m/z = 79$ amu) was monitored. Residual bromide ion concentration was determined in the aqueous phase of selected microcosms at the end of the study. The purpose was to determine if the loss of HBCD observed in the microcosms resulted with the release of bromide ions. Complete degradation of the 1250 ng of HBCD added to the microcosms would have resulted in the release of 938 ng of bromide (HBCD contains 75% bromine by weight). Residual bromine levels in selected microcosms after 119 days of incubation were as follows:

Microcosm	Nominal Concentration of Bromine added as HBCD (ng/g)	Measured Bromide Concentration in Soil (ng/g)
Aerobic Background Soil	—	3,080
Aerobic Test Soil	19	1,380
Anaerobic Background Soil	—	4,700
Anaerobic Test Soil	19	4,420

The background levels of bromide present in the background microcosms were 160- to 250-fold higher than the theoretical concentration that could be released from the amount of HBCD added. These background levels were too high to permit quantitation of bromide released from the HBCD.

DISCUSSION

The purpose of this study was to determine the environmental lifetime of HBCD under realistic environmental conditions. Laboratory microcosms were used to evaluate the transformation of HBCD in aerobic and anaerobic soils. Soil degradation processes are expected to play a major role in determining the environmental lifetime of HBCD since, upon release into the environment, the majority of HBCD is likely to partition into the soil or sediment compartments. In this study, HBCD loss was observed in both viable and abiotic soil microcosms although the rates were appreciably faster in the viable soils. Biologically mediated transformation processes (*i.e.*, biotransformation) accelerated the rate of loss of HBCD when compared to the biologically inhibited (*i.e.*, heat-treated) soils. No brominated degradation products were observed in either system.

Over the last several years a number of international protocols (EC 1996, UNECE 1998, UNEP 2000) have been put forth for the classification of chemicals as persistent (P), bioaccumulative (B), and toxic (T) [17, 18, 19]. The criteria for persistence in these initiatives includes half-lives in soil and sediments ranging from 120 to 180 days. In this investigation the resulting biotransformation half-lives for HBCD were determined to be 63 and 6.9 days in the aerobic and anaerobic soils, respectively. Based upon these results HBCD cannot be considered "persistent in the environment" since the half-lives in soil are clearly below the criteria specified by the various international protocols including the 120 days sediment value specified in the European Commission's Technical Guidance Document (EC 1996).

Limited information is available for the reactions of HBCD in the environment. However, in a separate soil degradation study conducted by Lickly and coworkers [20] they observed the aerobic degradation and mineralization of a similar type of cyclic, aliphatic halogenated fire retardant, FR-651A (mixture of pentabromochlorocyclohexane, tetrabromo-dichlorocyclohexane, and tribromotrichlorocyclohexane). They reported a soil half-life of ~11 days based upon disappearance of ^{14}C -FR-651A from soil. Complete degradation of ^{14}C -FR-651A was also observed with mineralization half-life on the order of 93 days.

An examination of the reactions of brominated aliphatic compounds that contain structural features similar to HBCD can also provide insight into the possible reaction pathways available for HBCD. Brominated aliphatic compounds are known to be susceptible to both hydrolytic and nucleophilic attack. The reactivity of halogenated aliphatic compounds increases in the order of $F < Cl < Br$. For example, the hydrolysis half-lives at pH 7 and 25 °C for methyl fluoride, methyl chloride, and methyl bromide are 1.1×10^4 , 340, and 20 days, respectively [21]. At environmental pH's, neutral and base catalyzed hydrolysis reactions are most important, and depend on the degree and patterns of halogen substitution [22].

Sulfur based nucleophiles can react rapidly with halogenated aliphatic compounds, thereby reducing their lifetimes in the environment. The half-life of 1-bromohexane in water at 25 °C was reduced from 20 days to approximately one day in 5 mM HS^- or 0.07 mM polysulfide (S_x^{2-}) [23]. Similarly, the half-life of 1,2-dibromoethane in water at 25 °C was reduced from 1,000 days to 4 days in the presence of 5 mM HS^- . Sulfide and polysulfides would be expected to be present in soils at low redox potentials. Thus, reaction of HBCD with these sulfur-based nucleophiles may partly explain the loss of HBCD in the soil studies.

Since HBCD contains three pairs of vicinal bromine atoms, the transformation of simple aliphatic compounds containing vicinal bromine atoms may provide insight into possible reaction pathways for HBCD. For example, 1,2-dibromoethane reacts with nucleophiles via both substitution and elimination reactions [21]. Reaction with HS^- via an "SN2" substitution reaction results in the formation of $HS-CH_2-CH_2-SH$, while an elimination reaction under alkaline conditions results in the formation of $H_2C=CHBr$. A combination of elimination and substitution reactions can result in the formation of a mixture of $HO-H_2-CH_2-OH$ and $H_2C=CHBr$. Similar mechanisms may be responsible for the loss of HBCD observed in this study.

Halogenated aliphatic compounds are susceptible to reductive dehalogenation reactions in natural reducing environments. The rates of reactions vary depending on the strength and electron affinity of the carbon-halogen bond and the stability of the carbon-radical

species resulting from the initial electron transfer that occurs [23]. Vicinal dehalogenation reactions are particularly important, and are dependent on the halogen atom. Half-lives for vicinal dehalogenation of 1,2-diiodoethane, 1,2-dibromoethane, and 1,2-dichloroethane are 0.6, 55, and 1.8×10^4 hours, respectively [23]. The rapid disappearance of HBCD in the anaerobic soil microcosms may be partly explained by reductive dehalogenation reactions.

QUALITY ASSURANCE

This study was conducted in accordance with Good Laboratory Practice Standards [24, 25, 26, and 27]. The study conduct and data generated were reviewed according to the procedures of the Quality Assurance Unit of Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan.

ARCHIVING

The data, protocol, protocol changes/revisions, and final report are archived at Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan.

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Table 1. Experimental Design for Aerobic and Anaerobic Soil Biodegradation Experiments

Reaction Mixture	Description	Analysis	Purpose
Viable Test	Soil + HBCD (in carrier solvent)	LC/MS	Evaluate role of biological processes in the transformation of HBCD in soil
Abiotic Test	Abiotic soil + HBCD (in carrier solvent)	LC/MS	Evaluate role of abiotic processes in the transformation of HBCD in soil
Viable Background	Soil + carrier solvent	LC/MS	Evaluate possible analytical interferences of sample matrix
Abiotic Background	Abiotic soil + carrier solvent	LC/MS	Evaluate possible analytical interferences of heated treated sample matrix

Note: 1) HBCD was added to soil mixtures at 25 ng/g based on the soil dry weight
2) Acetone was used as carrier solvent

Table 2. Measurements of pH and Redox Potential for Selected Anaerobic Soil Microcosms

Incubation Time (days):	9 ^a	16 ^a	23 ^a	29 ^a	37 ^a	42 ^a	120 ^b
pH	6.2	6.4	6.4	6.5	6.5	6.5	6.8
Viable Background	6.1	6.4	6.4	6.5	6.5	6.5	6.8
Eh (mV)	218 ± 43 ^c	215 ± 30	268 ± 33	203 ± 0	146 ± 20	186 ± 7	— ^d
Viable Background							

^aStabilization time following preparation of microcosms - prior to HBCD addition

^bIncubation time following HBCD addition

^c± 1 Standard deviation

^dNo measurement made

Table 3. Headspace Oxygen Measurements in Selected Aerobic Soil Microcosms

Incubation Time (days):	<i>Percent Oxygen</i>										
	14 ^a	21	29	36	44	48	65	73	80 ^a	101 ^a	115
Viable Background	5	19	21	20	21	21	— ^b	21	22	20	20
	4	20	18	18	18	17		12	5	11	15
Viable Test						21					21
						22					
Viable Test	—	18	—	—	—	20	20	—	3	—	—
		18				22	13		11		—
Abiotic Background						16					1
						9					19
Abiotic Background	—	15	—	—	—	—	19	—	—	—	—
		20					12				18
Abiotic Test	—	22	—	—	—	—	17	—	—	—	—
		18					17				16

^a Headspace gases in viable microcosms exchanged with laboratory air after 14, 80, and 101 days of incubation

^b No measurement made

Table 4. HBCD Concentrations in Aerobic Soil Microcosms

Nominal HBCD concentration: 25 ng/g

Day	Viable			Abiotic		
	HBCD ng/g ^a	% Decrease	Recovery Factor ^b (%)	HBCD ng/g	% Decrease	Recovery Factor ^b (%)
0	15.9 ± 1.3 ^c	0	44	18.0	0	44 ^d
1	13.0 ± 2.1	18	31	- ^e	—	—
7	11.5 ± 0.3	28	46	-	—	—
21	9.5 ± 0.3	40	71 ± 8	16.3 ± 2.5	9	97 ± 28
48	6.6 ± 0.1	58	70 ± 3	—	—	—
65	5.1 ± 1.8	67	54 ± 1 ^f	16.0 ± 0.3	11	74 ± 25
119	4.0 ± 1.1	75	94 ± 1	17.4 ± 7.5	3	87 ± 18

^aBased on dry weight of soil

^bPercent recovery factor (determined from matrix spike samples) used to correct reported HBCD value.

^c± 1 standard deviation

^dPercent recovery factor applied from viable, day 0 matrix spikes prepared and analyzed on the same day.

^eNo measurement made

^fPercent recovery factor applied from soil anaerobic matrix spikes prepared and analyzed on the same day.

Table 5. HBCD Concentrations in Anaerobic Soil Microcosms

Nominal HBCD concentration: 25 ng/g

Day	Viable			Abiotic		
	HBCD ng/g ^a	% Decrease	Recovery Factor ^b (%)	HBCD ng/g	% Decrease	Recovery Factor ^b (%)
0	11.0 ± 0.1 ^c	0	68	17.2 ± 2.5	0	46
1	8.0 ± 5.1	27	79	— ^d	—	—
7	8.5 ± 0.1	23	51	—	—	—
21	0.9 ± 1.3	92	56 ± 10	19.1 ± 3.1	0	50 ± 23
56	0.5 ± 0.6	95	57 ± 3	11.8 ± 1.3	31	54 ± 1
91	nd ^e	>95	72 ± 13	—	—	—
119	nd	>95	62 ± 7	6.9 ± 1.9	60	59 ± 4

^aBased on dry weight of soil

^bPercent recovery factor (determined from matrix spike sample) used to correct reported HBCD value.

^c± 1 Standard deviation

^dNo measurement made

^eNot detected – detection limit 0.5 ng/g

Table 6. Pseudo-First Order Rate Constants and Half-lives for Transformation of HBCD in Soil

Microcosm Description	<u>Biotransformation</u>		<u>Abiotic (Control)</u>	
	k (days ⁻¹)	t _{1/2} (days)	k (days ⁻¹)	t _{1/2} (days)
Aerobic Soil	0.011	63	0.0001	6900
Anaerobic Soil	0.10	6.9	0.0085	82

Note: Biotransformation rate constant determined by subtracting abiotic control rate constant from viable rate constant.

Viable rate constants used (see Appendix G): Aerobic soil – 0.011 days⁻¹

Anaerobic soil – 0.11 days⁻¹

Half-life (t_{1/2}) calculated from the relationship $t_{1/2} = \ln(2)/k$

Figure 1. Representative LC-MS Chromatograms for HBCD Analysis using APPI. **A)** 640.7 m/z; **B)** 79 m/z

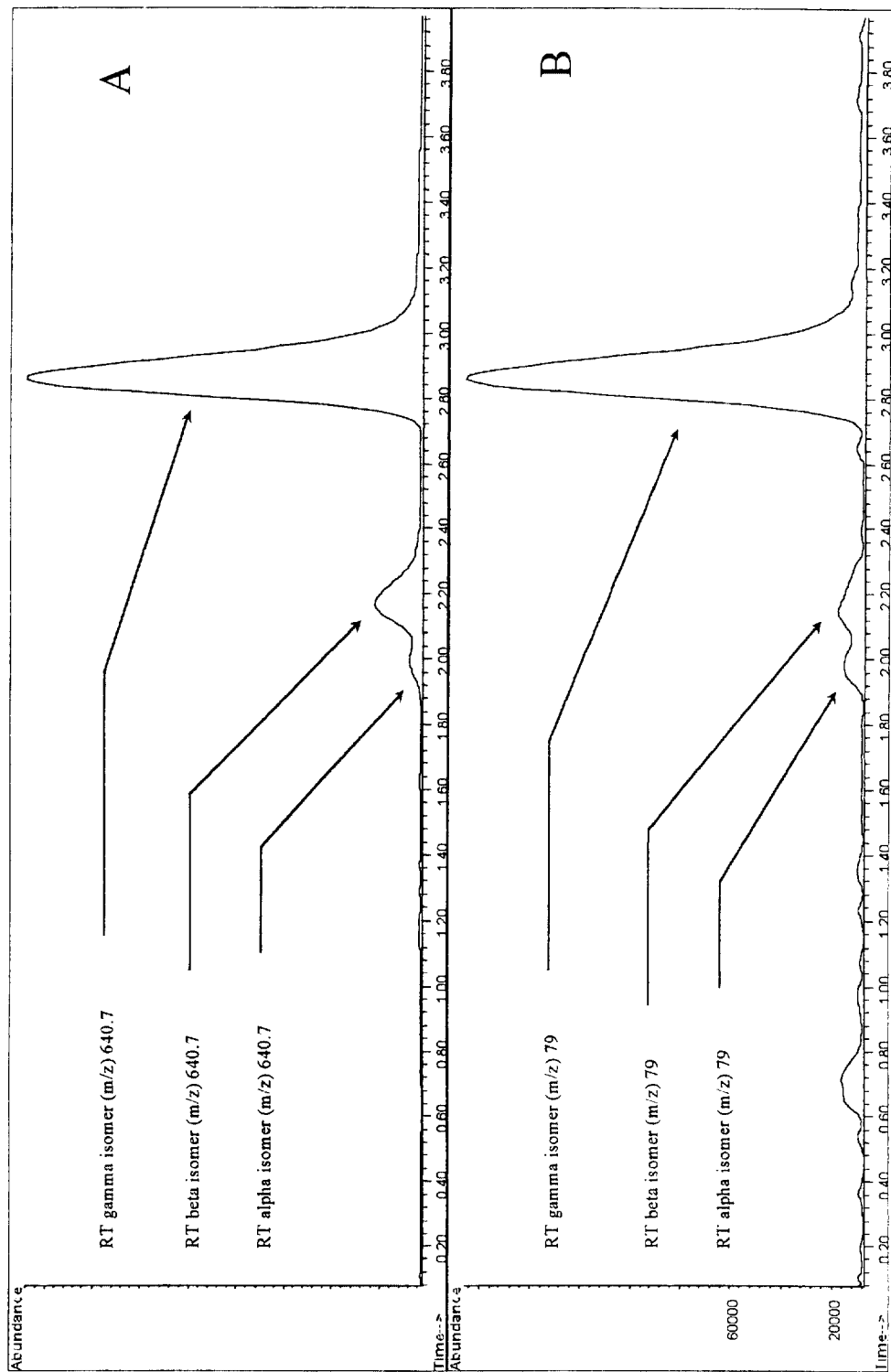


Figure 2. HBCD Concentrations in Aerobic Soil Microcosms

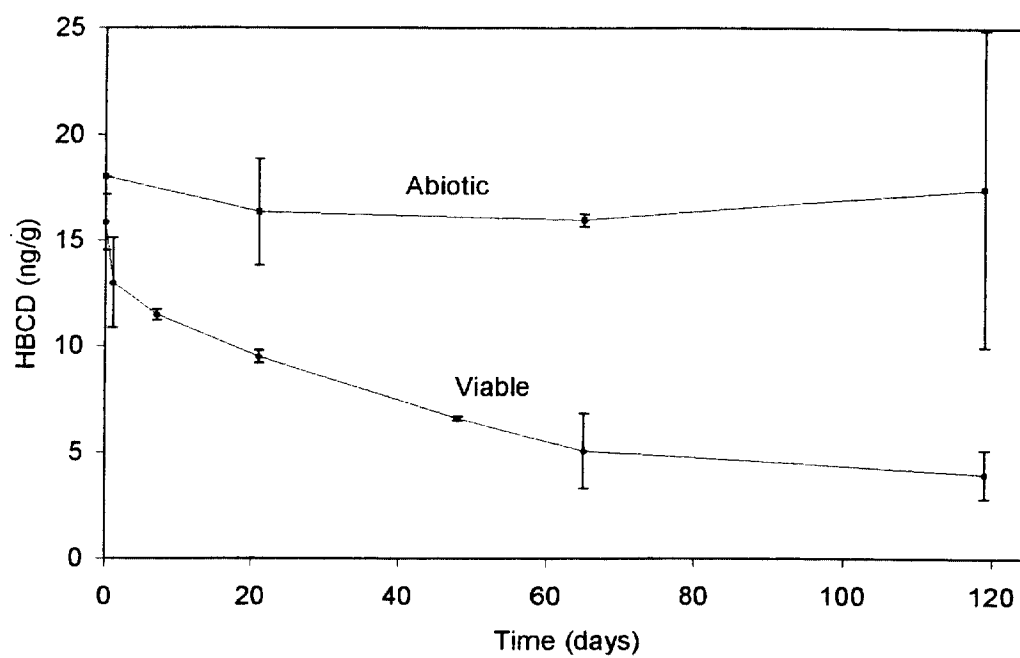


Figure 3. LC-MS Chromatogram for Day 0 Analysis of HBCD in Aerobic Soil Microcosm. A) 640.7 m/z; B) 79 m/z

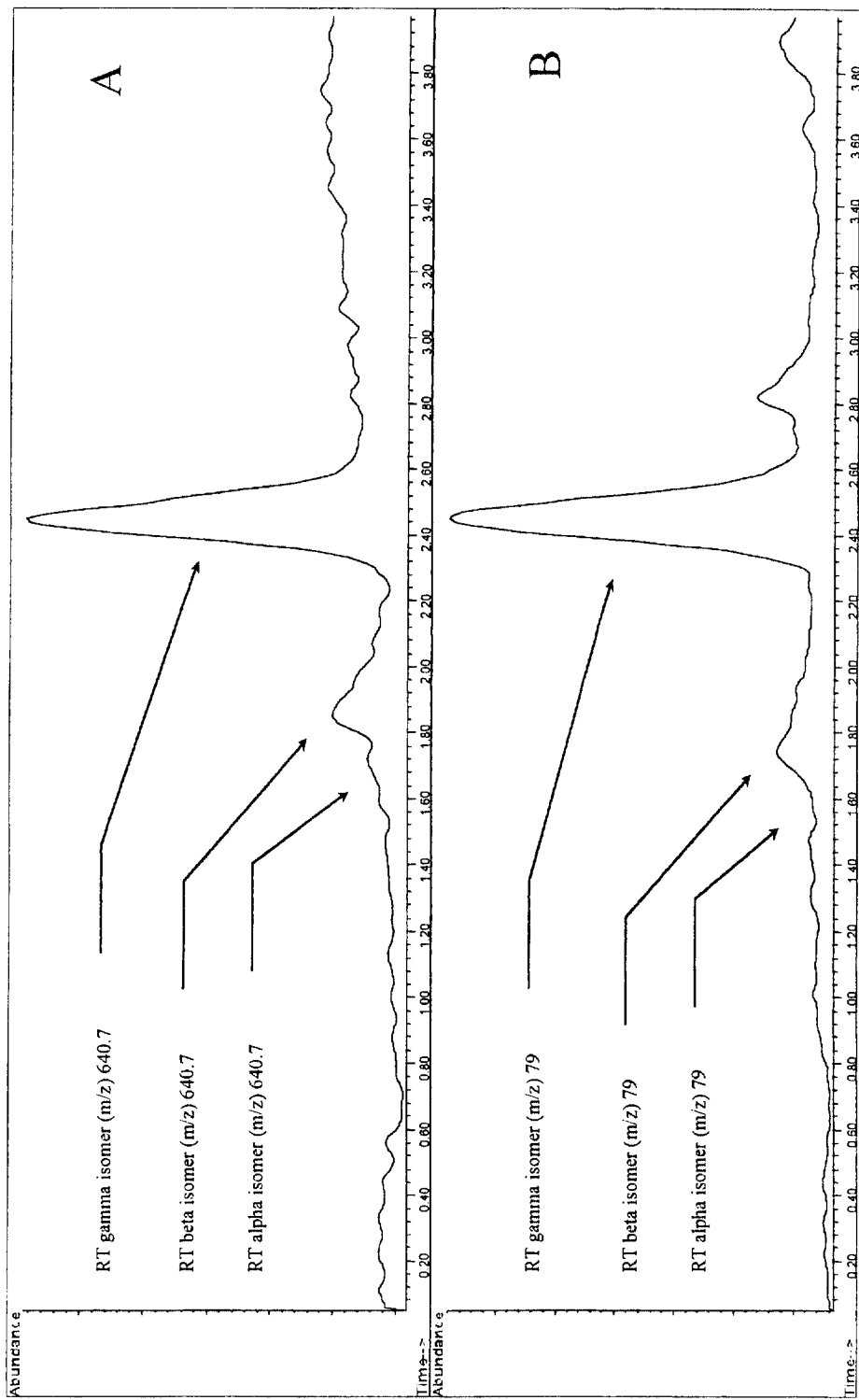


Figure 4. LC-MS Chromatogram for Day 65 Analysis of HBCD in Aerobic Soil Microcosm. **A)** 640.7 m/z; **B)** 79 m/z

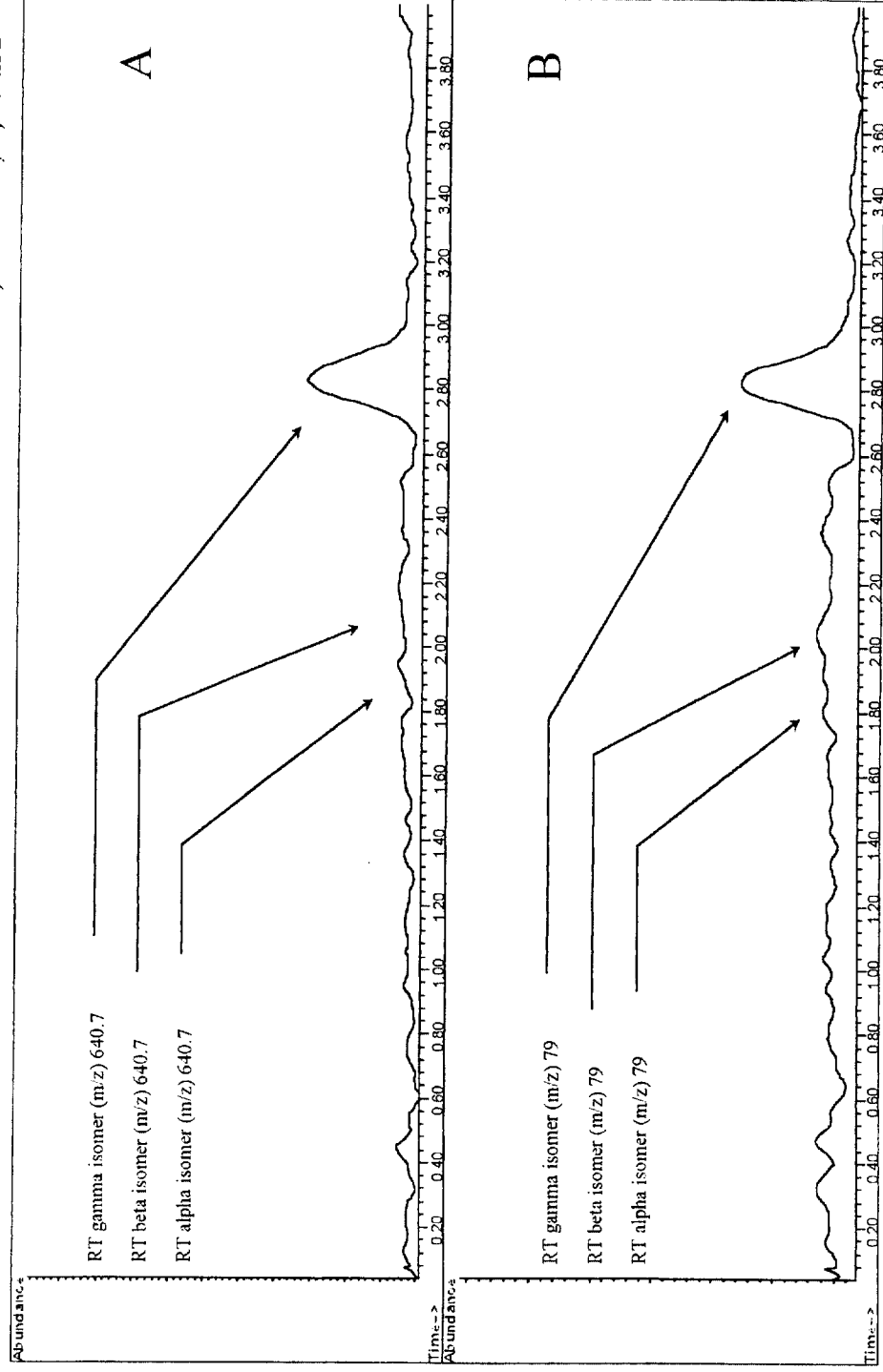


Figure 5. LC-MS Chromatogram for Day 119 Analysis of HBCD in Aerobic Soil Microcosm. A) 640.7 m/z; B) 79 m/z

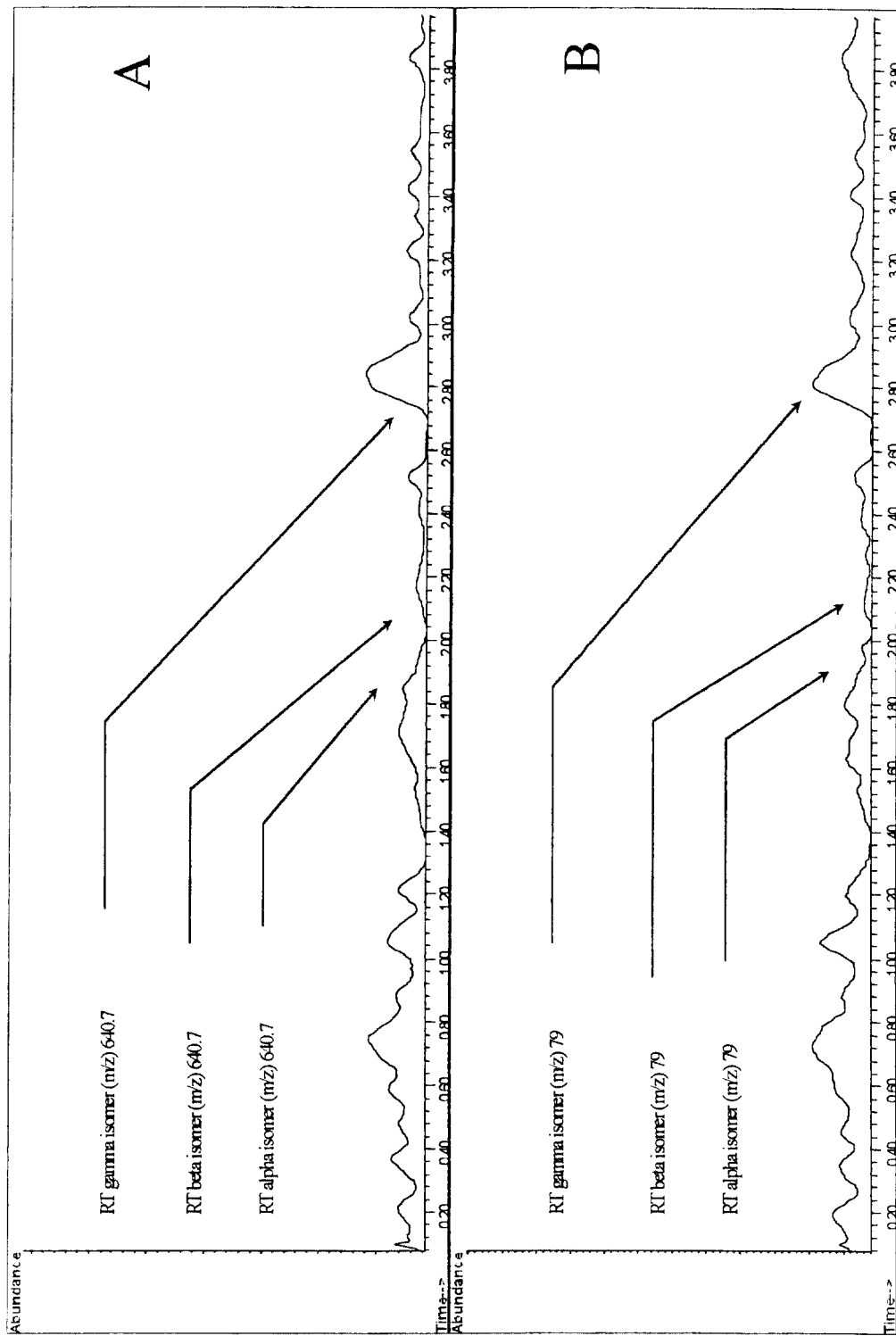


Figure 6. LC-MS Chromatogram for Day 119 Analysis of HBCD in Abiotic Aerobic Soil Microcosm. A) 640.7 m/z; B) 79 m/z

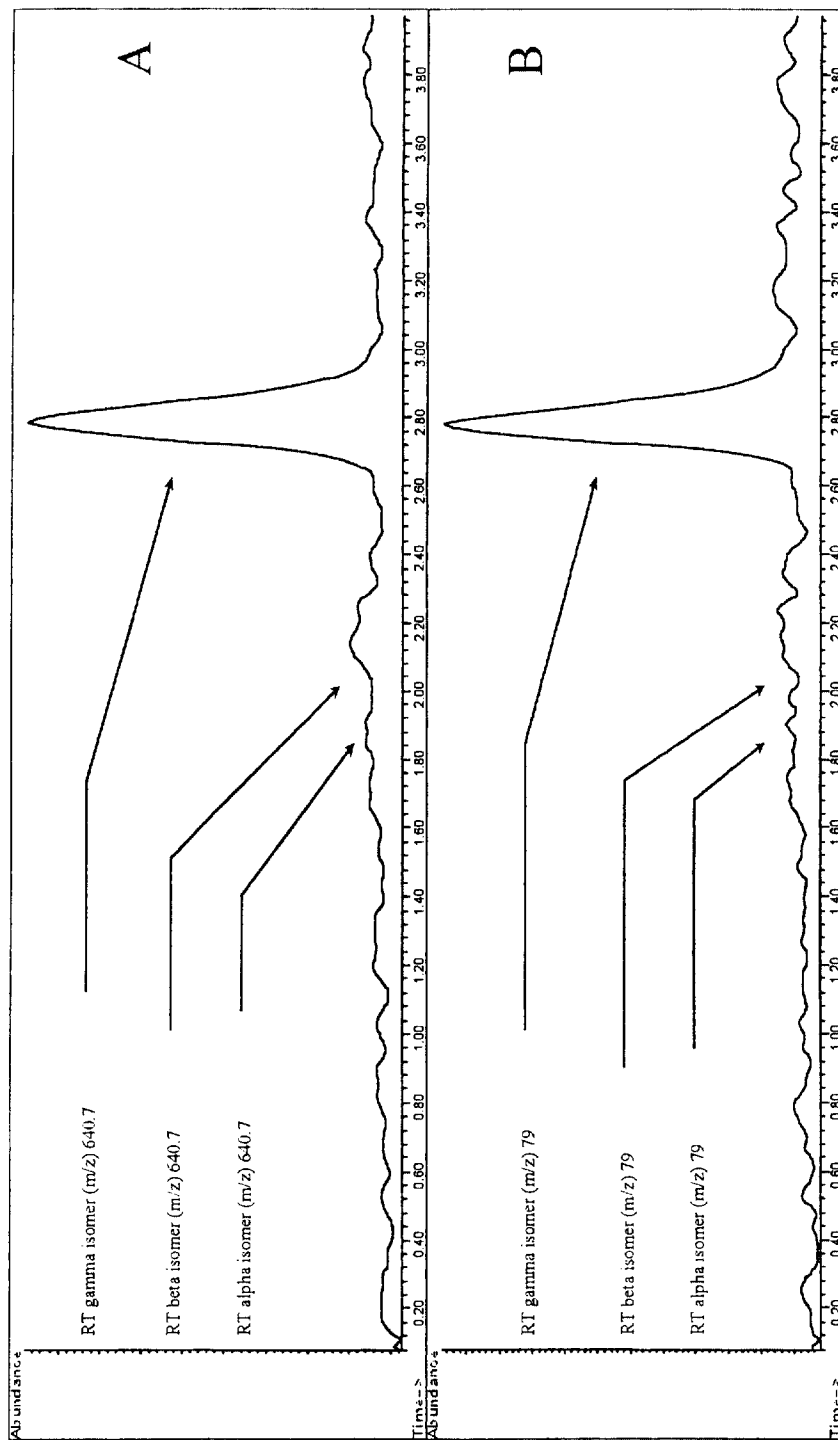


Figure 7. HBCD Concentrations in Anaerobic Soil Microcosms

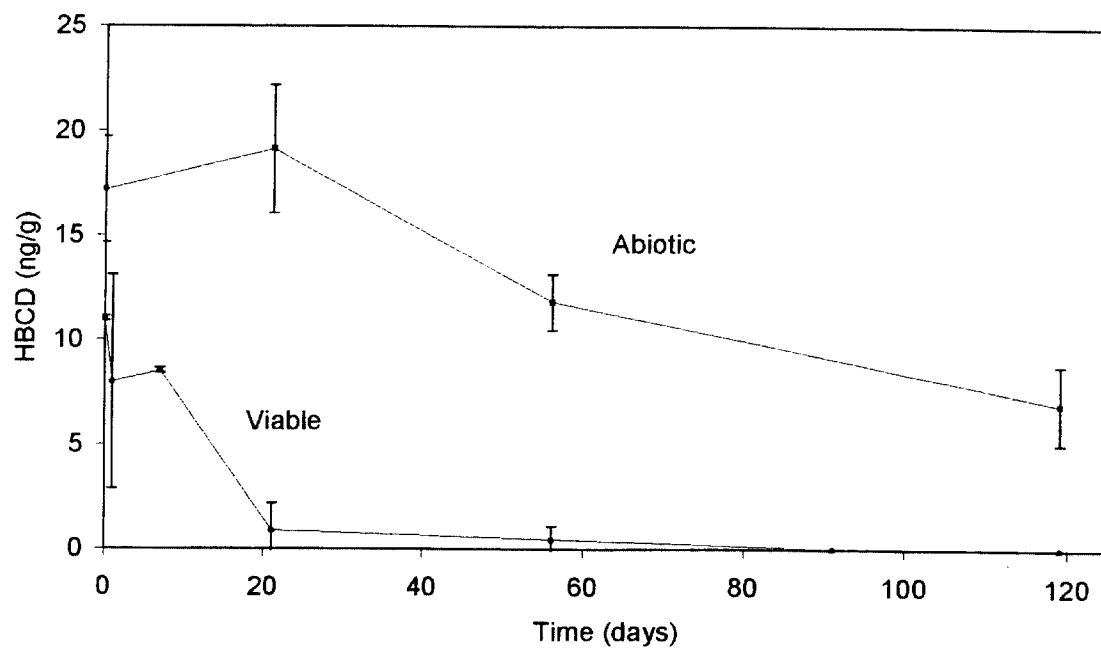


Figure 8. LC-MS Chromatogram for Day 0 Analysis of HBCD in Anaerobic Soil Microcosm. A) 640.7 m/z; B) 79 m/z

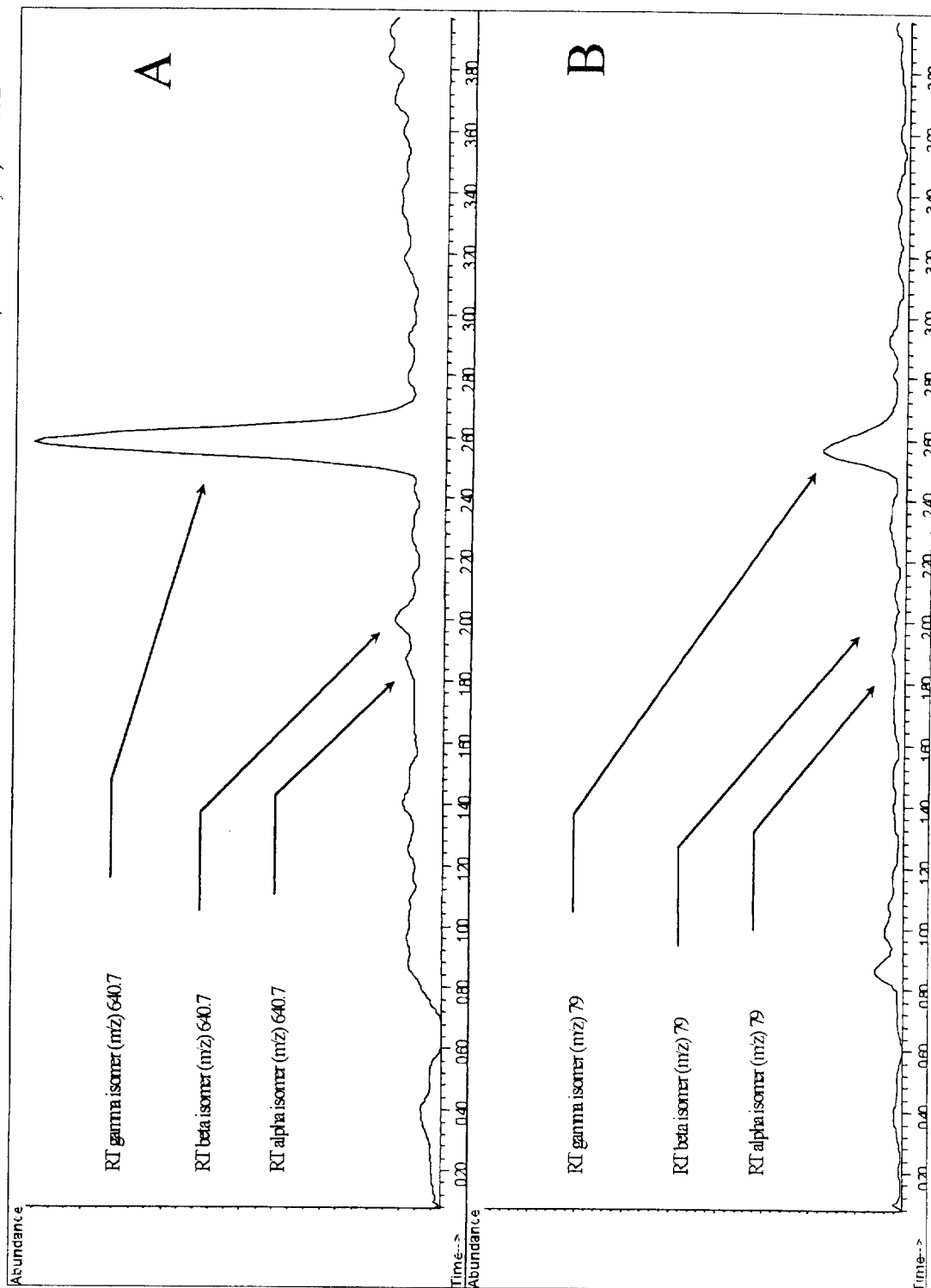
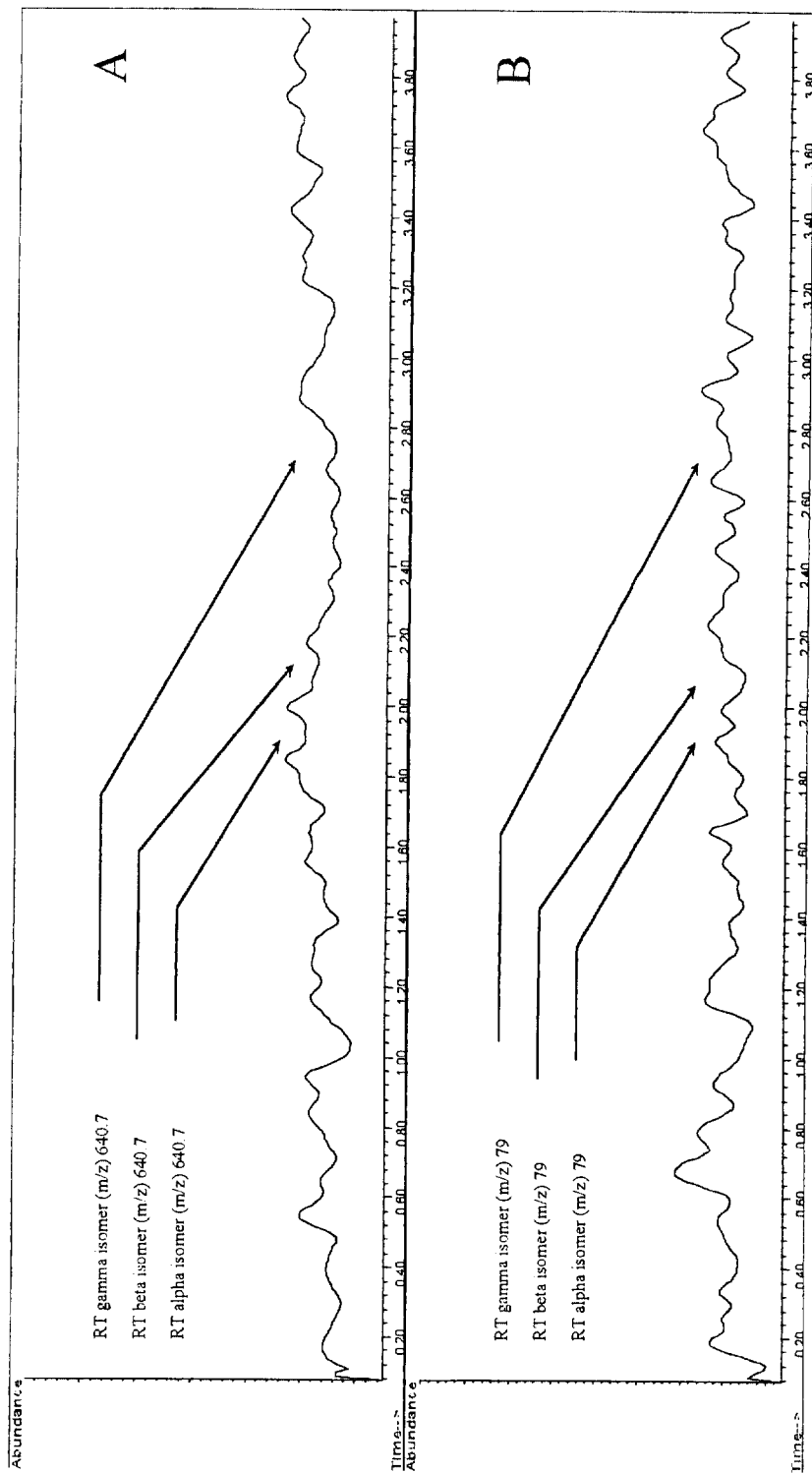


Figure 9. LC-MS Chromatogram for Day 119 Analysis of HBCD in Anaerobic Soil Microcosm. A) 640.7 m/z; B) 79 m/z



Appendix A. Preparation of Soil Microcosms for LC-MS analysis of HBCD

- 1) Dry soil sample with anhydrous sodium sulfate (25 g for aerobic microcosms and 75 g for anaerobic microcosms).
- 1) Add 100-mL hexanes to soil sample and mix 1 day on reciprocating shaker.
- 2) Centrifuge soil sample at 1000 rpm 10 minutes.
- 5) Hexanes extract of the soil sample is decanted into a 40 mL vial.
- 6) About 11 mL of the extract are transferred into a 20 mL vial containing 5 mL of Milli-Q® water. The remainder of the extract is saved as a retainer sample.
 - The vial is capped and shaken vigorously. This wash step removes residual salts from the hexanes extract (Note: Failure to wash the extract sample results in the accumulation of salts in the LC-MS interface, resulting in a rapid decrease in instrument sensitivity). The washed extract is allowed to stand for several minutes to allow for complete phase separation between the organic and water phase.
 - Using a 10 mL disposable glass pipette, a measured portion of the washed extract (typically 9.5 mL) is transferred into a 20 mL vial.
 - The washed extract is evaporated to dryness in 50 to 60 minutes at medium heat in a speed vac.
 - 1 to 2 mL of acidified diluent (acetonitrile/water (85/15) + 0.1% HCl) is added to the vial to dissolve the residue. The vial is capped and placed on a vortex mixer for approximately 1 minute. The reconstituted sample is transferred to a 2-mL vial, capped, and analyzed by LC-MS.

Appendix B - HBCD LC/MS Instrumentation and Conditions

Agilent 1100 Series LC/MS

- 1100-SL Mass Selective Detector - G1946D

- Group 1 – Select Ion Monitoring (SIM)

On at 0.0 minutes:

<u>SIM Ion</u>	<u>Fragmentor</u>	<u>Gain (EMV)</u>	<u>SIM Resolution</u>	<u>Dwell</u>
79	160	30.0	Low	289
640.7	160	30.0	Low	289

- Atmospheric Pressure Photo Ionization (APPI) Source: G1947-60101

- Gas Temp: 250 °C.
- Vaporizer Temp: 240 °C.
- Dry Gas: 7.0 L / minute.
- Nebulizer Pressure: 45 psig.
- VCap (negative): 5000 V

- Bin Pump – G1312A.

- Column Flow: 0.75 mL/min - Isocratic.
- Stop Time: 4 minutes.
- Solvent A-2: 15% Water + 0.1% Formic Acid.
- Solvent B-2: 85% Acetonitrile + 0.1% Formic Acid.

- Auto sampler – G1313A.

- Injection Volume: 100 µL.

- Column Oven - G1316A

- Temperature Setting: Ambient.

- Variable Wavelength Detector (VWD) – G1314A.

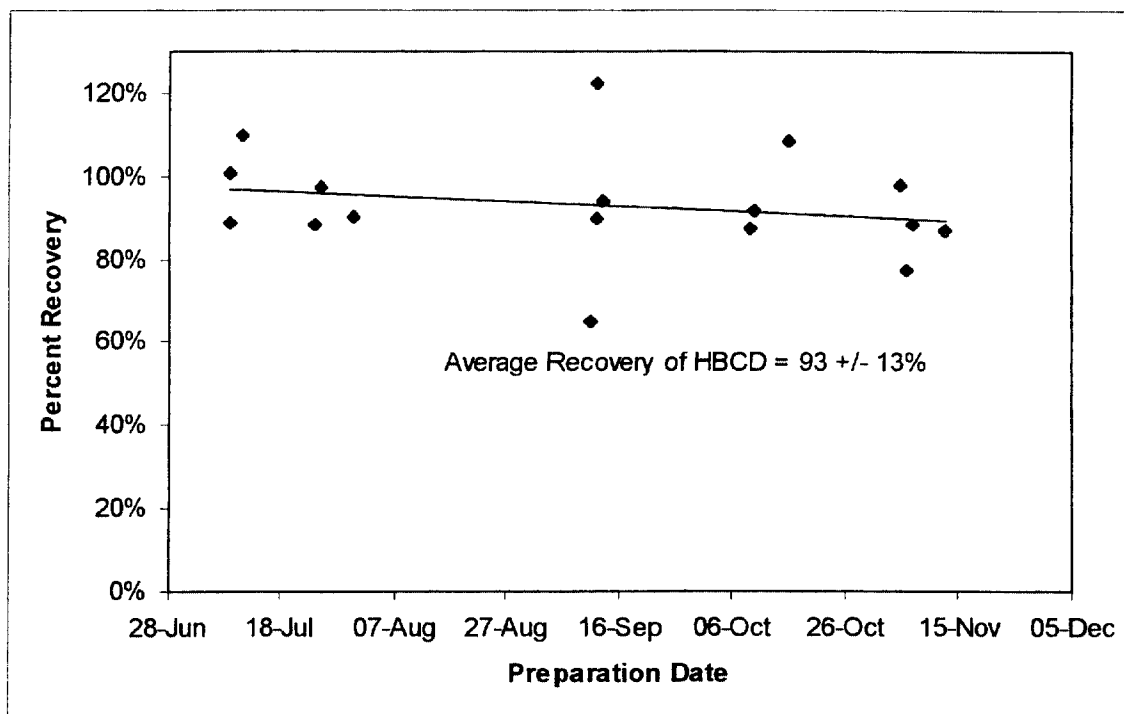
- Wavelength: 254 nm.
- Peak width: > 0.1 minute.

Hitachi Model L-6200A LC (doping pump) – Pump Flow: 0.1 mL/minute.

Column: ODS-AQ 5µm; 2 x 150 mm

Data Acquisition: Agilent LC/MSD ChemStation – Rev A.08.04 [1008]

Appendix C. Recovery of HBCD in Method Spike Samples Over Time.



Appendix D. Gas Chromatography-Mass Spectrometry Instrument Conditions

Instrumentation

Agilent 6890 Gas Chromatograph (GC) -
Column: Agilent DB-624; 0.32 mm x 30m; 1.8 μ m film
CTC Analytics – Leap GC Pal Auto sampler
Agilent 5973 Mass Selective Detector (MSD)

GC Conditions:

Injection Port

Injection volume (1 μ L)
Splitless mode
Injection Port Temperature – 250 °C
Pressure: 10 psi (helium carrier gas)
Purge Flow: 30 mL/min
Purge Time: 1 minute
Total Flow: 36.4 mL/min

Temperature Program

40 °C hold for 1 minute
Ramp @ 15 °C/minutes to 200 °C
Hold 200 °C for 4 minutes

MSD conditions:

Solvent delay: 1 minute
EM offset: 200
Group 1 ions – low resolution (m/z): 79 and 81; dwell = 50
MS Quad temp: 150 °C
MS Source temp: 230 °C

Appendix E. Ion Chromatograph Conditions for Inorganic Bromide Analysis

Instrument: Dionex Model DX-120 Ion Chromatograph

Column: Dionex AS9-HC-4 mm (10-32)

Auto Sampler: Alcott Model 728R

Injection volume: 20 μ L

Mobile phase: 11 mM Na_2CO_3

Flowrate: 1.25 mL/minute

Pressure: 2476 psi

Total conductivity: 23.47 μ S

Offset: 0.076

Eluent: 11 mM Na_2CO_3

Perkin-Elmer TurboChrom[®] Data Acquisition System

Appendix F. Gas Chromatography-Flame Ionization Detection Instrument Conditions for Methane Analysis

Gas Chromatograph: Agilent model 6890

Column: PoraPlot Q-HT 0.53 mm x 25 m

Injection Port: 200 °C

Split mode, 50 cc/min split vent

7.5 p.s.i. head pressure

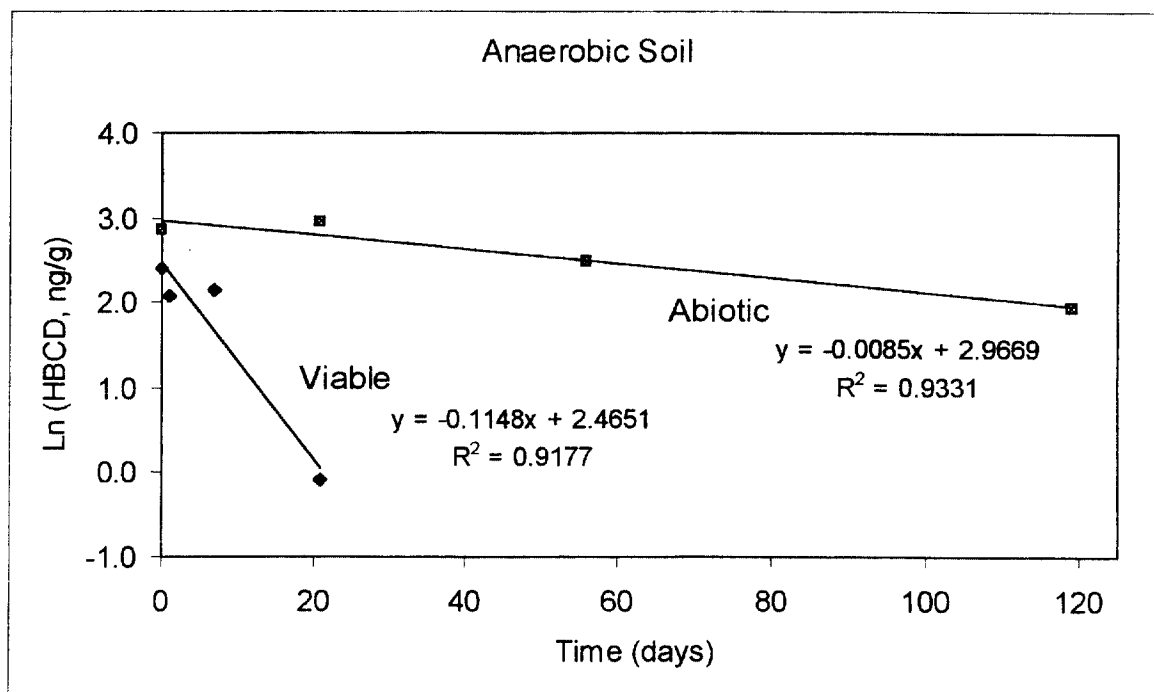
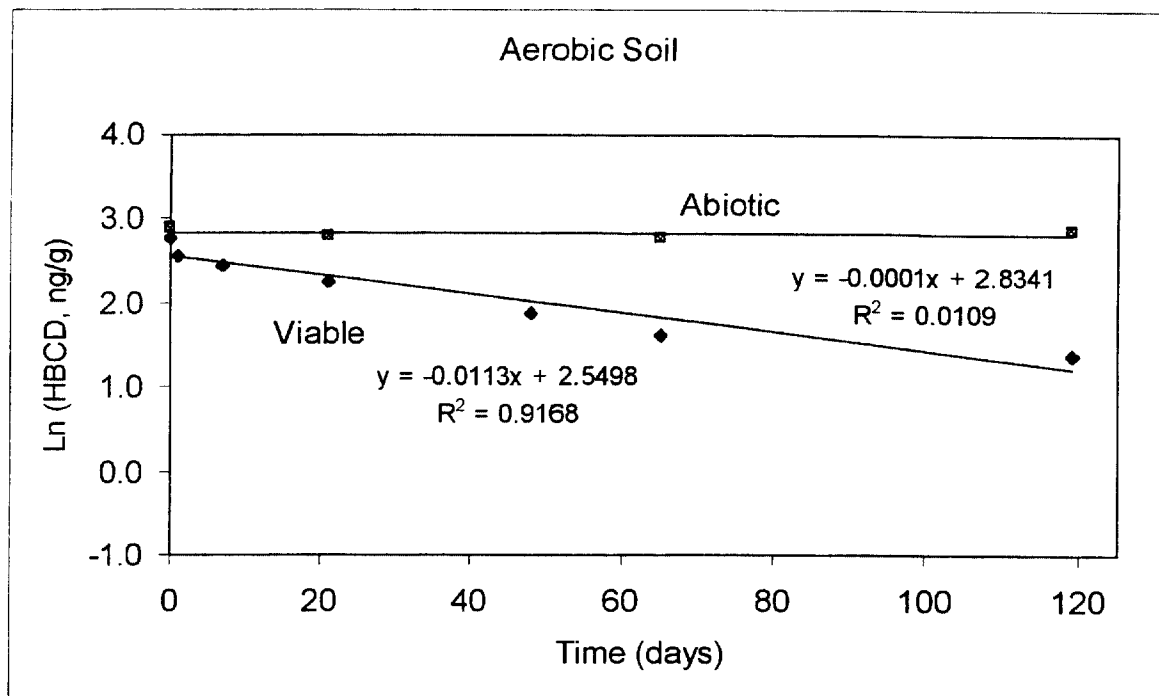
Carrier gas: Helium

Column: 45 °C isothermal

Flame Ionization Detector: 250 °C

Perkin-Elmer TurboChrom® Data Acquisition System

Appendix G. Plots of Natural Logarithm of HBCD Concentration versus Time



Appendix H Protocol and Protocol Change/Revisions

Final Protocol

TOXICOLOGY & ENVIRONMENTAL RESEARCH AND CONSULTING
THE DOW CHEMICAL COMPANY

PROTOCOL
ENVIRONMENTAL CHEMISTRY RESEARCH LABORATORY
1803 BUILDING, MIDLAND, MICHIGAN 48674

TITLE: EVALUATION OF AEROBIC AND ANAEROBIC TRANSFORMATION OF
HEXABROMOCYCLODODECANE IN SOIL

KEY NUMBERS

FILE #: DR-0099-6827-004
STUDY ID: 021082
PTR: 60143-428-2
CAS: 25637-99-4

PLANNED DATES

EXPERIMENTAL START: June 24, 2002
EXPERIMENTAL TERMINATION: November 28, 2002
ESTIMATED FINAL REPORT: February 28, 2003
GLP STUDY: Yes

SPONSOR: American Chemistry Council's
Brominated Flame Retardant Industry Panel
1300 Wilson Boulevard
Arlington, Virginia 22209

REQUIRED SIGNATURES:

STUDY DIRECTOR: S. J. Gonsior 20 June 2002
S. J. Gonsior, M.S. /DATE

MANAGEMENT APPROVAL: K. A. Brooks 20 June 2002
K. A. Brooks, B.S. /DATE

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OBJECTIVE

The objective of this study is to assess the fate and transformation of hexabromocyclododecane (HBCD) under both aerobic and anaerobic conditions in soil. The experimental procedure will be based on a modification of the Organisation for Economic Co-Operation and Development (OECD) proposed Guideline 307 "Aerobic and Anaerobic Transformation in Soil" [1].

INTRODUCTION

HBCD is used as a flame retardant in several applications, including expanded and extruded polystyrene. HBCD has the potential for release into the environment from production processes, and from the processing and disposal of fabricated products containing the compound.

Biodegradation is a major process for removing chemicals from the environment. Biodegradation can take place under aerobic or anaerobic conditions. Aerobic biodegradation processes can predominate in surface waters, surface soils, and the aeration basins of wastewater treatment plants (WWTPs). Anaerobic processes, in contrast, can occur in aquatic sediments, groundwater, and anaerobic digestion units in WWTPs. Little information is available concerning the aerobic and anaerobic biodegradability of HBCD. The compound has been reported not to be readily biodegradable based on the results of an OECD 301D Closed Bottle Test [2]. In this study, no degradation of 7.7 mg/L HBCD was observed in 28 days. Note that the test concentration of HBCD exceeded the reported water solubility of 3.4 µg/L by greater than 2000-fold. The lack of measurable biodegradation in this test was due, in part, to the limited availability of the test material to the microorganisms as well as the low amount of biomass contained in the inoculum.

Aerobic and anaerobic biodegradation studies will be conducted in microcosms prepared with a representative surface soil. Concentrations of HBCD and any detected brominated degradation products will be determined using a sensitive and compound-specific analysis such as liquid

chromatography/mass spectrometry (LC/MS). The analysis will allow the primary biodegradation of HBCD to be followed and degradation rates to be determined.

MATERIALS

Test Material

A 25-gram sample of HBCD was obtained from Wildlife International, Ltd., Easton, Maryland. The sample was identified as a "Representative Sample of Composite of HBCD – WIL# 5850," with a lot number MC258500102. The sample is a composite of test materials supplied by Albemarle Corporation, Great Lakes Chemical Corporation, and Eurobom B.V. The sample has been characterized by Albemarle Corporation. Results of this analysis will be included in the study file and final report. The molecular formula for HBCD is $C_{12}H_{18}Br_6$ ($M_w = 641.7$). The vapor pressure of the compound is reported to be 6.27×10^{-5} Pa at 21 °C [2].

All other chemicals will be obtained from commercial sources, with appropriate documentation of identity and purity. Water will be purified in a MilliQ® Water Purification System (Millipore Corporation, New Bedford, Massachusetts).

Soil Sample

Agvise Laboratories (Northwood, North Dakota) collected a representative sandy loam soil (USDA classification) on April 16, 2002. The soil sample was dispensed into seven metal cans, packed with blue ice, and shipped via overnight air express to The Dow Chemical Company. The soil sample was passed through a 2-mm sieve to remove stones, plant matter and to improve sample homogeneity and stored at approximately 4 °C until used.

Agvise Laboratories characterized a sub-sample of the collected soil. The soil was classified as a sandy loam (United States Department of Agriculture classification system) with 64% sand; 20% silt, and 16% clay. The pH was 6.6, cation exchange capacity 19.2 meq/100 g, bulk density 1.11 g/cc, and moisture at 1/3 bar 23.9 percent. The percent organic carbon by combustion was 1.8%. Microbial biomass content was determined to be 274 µg/g dry weight soil.

EXPERIMENTAL METHODS

Test Procedure – Aerobic Soil Reaction Mixtures

A series of soil microcosms will be prepared by adding portions of soil (*e.g.*, 50 g dry weight) to 250-mL serum bottles. The moisture content of soil will be adjusted to approximately 20% by weight by the addition of water to the microcosms. This moisture content is approximately 50 percent of the water holding capacity of a typical sandy loam soil in the United States (Annex 3 of OECD Guideline 307). The guidelines state that the moisture content should be in the range of 40 to 60 percent of the water holding capacity. The serum bottles will be sealed with Teflon-coated rubber septa contained in screw-on caps. The reaction mixtures will be stored at approximately 20 °C for approximately two to four weeks (pre-incubation period) to re-establish equilibrium of microbial metabolism following the change from sampling and storage conditions to test incubation conditions [1]. The OECD guidelines recommend a range of two days to four weeks for this pre-incubation period.

Following the pre-incubation period, the screw on caps will be removed and the headspace gases of the microcosms will be allowed to exchange with fresh air. HBCD will be added to replicate test mixtures at an approximate nominal concentration of 10 µg/kg (based soil dry weight [3]) as described in Table 1. Acetone will be used as a carrier solvent to introduce the HBCD into the microcosms. The volume of carrier solvent used will be minimized to reduce the potential for inhibitory effects on the microorganisms present. [Note: If adequate analytical sensitivity for HBCD cannot be achieved following method development in the soil matrix, the initial concentration of HBCD added to the reaction mixtures will be increased to ensure that the degradation/removal of HBCD can be followed through at least two half-lives]. A quantity of activated sludge (5 mg of mixed liquor suspended solids per g of dry soil [4]) will be added to the microcosms to simulate the addition of the test material to soil as a result of sewage sludge/farming application [1]. The soil mixtures will be mixed with a spatula to evenly distribute the HBCD and sludge in the microcosms. The microcosms will be left unsealed for approximately one hour to

allow the acetone to evaporate. Biologically inhibited controls will be prepared by heat sterilization prior to the addition of the test compound. The activated sludge added to the biologically inhibited controls will also be autoclaved. Blank mixtures and biologically inhibited blank and control mixtures will be prepared to correct for possible analytical interferences and abiotic losses of the test material (Table 1).

Test Procedure – Anaerobic Soil Reaction Mixtures

Microcosms for the anaerobic transformation experiment will be prepared in a similar manner as for the aerobic experiment, except that the soil will be covered with water and oxygen removed from the reaction mixtures to allow anaerobic conditions to develop. A series of soil microcosms will be prepared by adding portions of soil (50-g dry weight) to 250-mL serum bottles and covering the soil with 20-mL water. The microcosms will be transferred to an anaerobic glove box to allow residual oxygen to be removed. The anaerobic glove box contains an atmosphere equilibrated from a gas mixture containing ~70% (vol) N₂, ~28% CO₂, and ~2% H₂. Thereafter, the microcosms will be sealed and incubated in the in the glove box at approximately 20 °C for up to four weeks to allow methanogenic conditions to develop. Selected microcosms will be analyzed for pH, redox potential, and methane gas formation once per week. Resazurin, a redox dye, will be added to a subset of microcosms at 1 mg/kg to indicate when the redox potential of the reaction mixtures drops below –110 mV.

When anaerobic conditions are confirmed (*i.e.*, methane formed and redox potential <-110 mV), test microcosms will be spiked with HBCD according to the experimental design described in Table 1. A quantity of activated sludge (5 mg of mixed liquor suspended solids per g of dry soil) will be added to the microcosms to simulate the addition of the test material to soil as a result of sewage sludge/farming application [1]. Biologically inhibited controls will be prepared in the same manner as the test microcosms, except that the microcosms will be heat sterilized prior to the addition of the test material. The activated sludge added to the biologically inhibited controls will also be heat sterilized.

Microcosm Incubation

Both aerobic and anaerobic microcosms will be incubated in the dark at 20 ± 2 °C. Microcosms will be sacrificed for analysis according to the sampling schedule shown in Table 2. The sampling schedule may be adjusted based on initial results. The duration of the experiment will not exceed three months.

Analytical Methods

A liquid chromatography/mass spectrometry (LC/MS) analysis will be used to measure HBCD concentrations. This methodology was previously developed for the analysis of HBCD in water and fish tissues, and will be modified and optimized for use in this study [5,6]. At selected incubation intervals, duplicate test mixtures will be sacrificed for extraction and analysis of HBCD. One blank mixture will be extracted and analyzed for potential background interferences, and a second blank mixture will be spiked with 10 µg/kg HBCD (based on soil dry weight), extracted and analyzed for HBCD to determine the recovery of HBCD from the soil. Biologically inhibited controls and blanks will be analyzed in a similar manner according to the schedule shown in Table 2. The extraction procedure will involve adding an immiscible organic solvent (*e.g.*, hexanes) through the septum, and shaking and/or sonicating the sample to facilitate extraction of sorbed HBCD into the organic solvent. When detected, brominated degradation products from HBCD will be tentatively identified and quantified by LC/MS. Gas chromatography/mass spectrometry (GC/MS) may also be used to identify brominated degradation products and evaluate possible interferences in the sample matrices. The headspace gases in selected microcosms will be analyzed to determine if volatile, brominated degradation products are formed. Confirmation of identity and concentrations of the degradation products will likely depend on the availability of commercial standards. Sample extraction procedures, LC/MS and GC/MS conditions will be documented in the final report.

DATA AND REPORTING

Biodegradation of HBCD

Concentrations of HBCD and any brominated degradation products that are detected in the viable and biologically inhibited control reaction mixtures will be reported over time. Percent biodegradation (primary) for HBCD will be reported based on a comparison of measured HBCD concentrations at time t to HBCD concentrations measured on day 0. Loss of HBCD due to biodegradation will be determined by subtracting the rate of loss in biologically inhibited controls from the rate of loss observed in viable mixtures.

The final report will contain information on the test material, soil sample, test conditions, and interpretation of results as described in the test procedure [1].

Statistical Methods

Descriptive statistics (mean, standard deviation) will be used as appropriate to evaluate and report results from this study.

QUALITY ASSURANCE AND SAFETY

This study will be conducted in accordance with Good Laboratory Practice Standards [7,8,9,10]. The study conduct and data generated will be reviewed according to the procedures of the Quality Assurance Unit of Toxicology & Environmental Research and Consulting, The Dow Chemical Company. Permanent records of all data generated during the course of this study, the protocol, protocol revisions/changes, and the final report will be available for inspection by the Quality Assurance Unit of Toxicology & Environmental Research and Consulting, The Dow Chemical Company. All data generated including the protocol, protocol revisions/changes, and final report will be archived at the Toxicology & Environmental Research and Consulting, The Dow Chemical Company.

Laboratory procedures will be conducted in accordance with The Dow Chemical Company Research and Development EH&S Resource Manual. Routine safety precautions will include wearing gloves, safety glasses, and lab coats. In addition, specific safety information will be made available, and all

personnel involved in the study will be advised of the safety precautions to follow prior to handling the test material and working in the laboratory.

REFERENCES

- 1.) Organisation for Economic Cooperation and Development Guidelines for Testing of Chemicals, Proposal for a New Guideline 307, Aerobic and Anaerobic Transformation in Soil (2000).
- 2.) Draft Risk Assessment for Hexabromocyclododecane. March 5, 1999.
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- 4.) Tchobanoglous, G and Burton, F. L. Land Application of Sludge. Wastewater Engineering. Treatment, Disposal, and Reuse. 3rd Edition. Metcalf & Eddy, Inc.
- 5.) Wildlife International, Ltd. (2001). The Analysis of Hexabromo-cyclododecane (HBCD-as Separate Alpha, Beta, and Gamma Diastereomers) Concentrations in Support of Wildlife International, Ltd. Project Number 439A-112. Appendix 5.
- 6.) Wildlife International, Ltd. (2000). The Analysis of Hexabromo-cyclododecane (HBCD) Concentrations in Freshwater/Rainbow Trout Tissue in Support of Wildlife International, Ltd. Project Number 439A-111. Appendix 5.
- 7.) U.S. Environmental Protection Agency. Toxic Substances Control Act: Good Laboratory Practices Standards. 40 CFR Part 792, Final Rule.
- 8.) OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring, Number 1. OECD Principles on Good Laboratory Practice (as revised in 1997) ENV/MC/CHEM(98)17.
- 9.) EC Directive 99/11/EC of 8 March 1999 (OJ No. L 77/8-21, 23/3/1999).
- 10.) MHW/METI 59 KIKYOAU 85; EA, KANKIKEN No. 233; MHW, EISEI No. 38 and METI, 63 KIKYOAU No. 823.

Table 1. Experimental Design for Aerobic and Anaerobic Soil Biodegradation Experiments

Reaction Mixture	Description	Analysis	Purpose
Blanks	Soil mixtures + carrier solvent	LC/MS	Evaluate possible interferences in sample matrix
Test Mixtures	Soil mixtures + 10 µg/kg HBCD (in carrier solvent)	LC/MS	Determine transformation rates of HBCD in soil
Biologically Inhibited Blanks	Heat sterilized soil mixture + carrier solvent	LC/MS	Evaluate possible interferences in biologically inhibited sample matrix
Biologically Inhibited Controls	Heat sterilized soil mixture + 10 µg/kg HBCD (in carrier solvent)	LC/MS	Determine abiotic degradation rates for HBCD in soil

Note: HBCD will be added to soil mixtures at 10 µg/kg based on the soil dry weight

Table 2. Sampling Schedule for Aerobic and Anaerobic Soil Biodegradation Experiments

Day	Blanks*	Test Mixtures	Number of Microcosms Analyzed			Total Number of Samples
			Biologically Inhibited Blanks*	Biologically Inhibited Controls		
0	2	2	2	2		8
1	2	2	-	-		4
7	2	2	-	-		4
14	2	2	2	2		8
21	2	2	-	-		4
28	2	2	2	2		8
56	2	2	-	-		4
90	2	2	-	-		4
120	2	2	2	2		8
Total:	18	18	8	8		52

* For each sampling day, one blank mixture and one biologically inhibited blank mixture will be spiked with 10 µg/kg of HBCD (based on soil dry weight) to determine recovery of HBCD from sample matrix.

PROTOCOL CHANGE/REVISION

TOXICOLOGY & ENVIRONMENTAL RESEARCH AND CONSULTING
THE DOW CHEMICAL COMPANY, 1803 BUILDING, MIDLAND, MICHIGAN 48674

Study Title: EVALUATION OF AEROBIC AND ANAEROBIC TRANSFORMATION OF
HEXABROMOCYCLODODECANE IN SOIL

Study ID: 021082

CHEC File Number: DR-0099-6827-004

Change(s)/Revision(s) Number: 1

CHANGE(S)/REVISION(S):

- 1) Pre-incubation or stabilization period for aerobic microcosms was 5 weeks, rather than the range of 2 to 4 weeks described in the protocol.
- 2) Pre-incubation or stabilization period for anaerobic microcosms was 6 weeks, rather than the range of up to 4 weeks described in the protocol.
- 3) Nominal HBCD concentration added to microcosms was increased from 10 to 25 ng/g (soil dry weight).
- 4) The duration of the soil studies was 4 months (119 days), not the 3 months described in the protocol (page 6).
- 5) The number of sampling days for both the aerobic and anaerobic soil studies was reduced from 9 to 7 sampling days.
- 6) Matrix spikes were spiked with 25 ng/g of HBCD rather than the 10 ng/g described in the protocol.
- 7) Beginning with the 21-day sampling, both background microcosms were spiked with HBCD as matrix spikes, rather than analyzed as single blank and matrix spikes as described in the protocol.

REASON(S) FOR CHANGE(S)/REVISION(S):

- 1) Additional time was required to develop and validate analytical method for HBCD.
- 2) Reason #1 and additional incubation time also needed for anaerobic microcosms to develop anaerobic (i.e. methanogenic) conditions.
- 3) Higher HBCD concentration required to follow removal of HBCD through at least 2 half-lives, as noted in protocol.
- 4) The 3-month value in the protocol was incorrect. Table 2 in the protocol contained the correct value of 120 days. The OECD 307 guideline on which study was based recommends 4 months.
- 5) The sampling schedule was adjusted based on the results of initial analyses. The revised schedule was sufficient to define the rate of removal of HBCD. The OECD guidelines recommend at least 6 sampling points.

- 6) The concentration of HBCD added to the matrix spikes was increased to match the higher initial dosing of the test microcosms.
- 7) Analysis of 2 matrix spikes on a sampling day provided improved precision for quantitation of HBCD concentrations in the microcosms.

IMPACT ON STUDY:

1-7) None

PLEASE FILE THIS CHANGE/REVISION WITH THE PROTOCOL.

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<i>K. A. Brooks</i>	<i>05 February 2003</i>
K. A. Brooks, B.S.	/DATE
MANAGEMENT APPROVAL	

<i>J. W. Davis</i>	<i>05 February 2003</i>
J. W. Davis, Ph.D.	/DATE
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STUDY TITLE

EVALUATION OF AEROBIC AND ANAEROBIC TRANSFORMATION OF
HEXABROMOCYCLODODECANE IN AQUATIC SEDIMENT SYSTEMS

Author(s)

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Study Completion Date

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Laboratory Project Study ID

021081

COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

Compound: HEXABROMOCYCLODODECANE

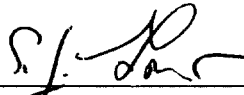
Title: EVALUATION OF AEROBIC AND ANAEROBIC
TRANSFORMATION OF HEXABROMOCYCLODODECANE IN
AQUATIC SEDIMENT SYSTEMS

All phases of this study were conducted in compliance with the following Good Laboratory Practice Standards:

US Environmental Protection Agency-TSCA GLPS
Title 40 CFR, Part 792-Toxic Substances Control Act (TSCA); Good Laboratory Practice
Standards, Final Rule


OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring,
Number 1. OECD Principles on Good Laboratory Practice (as revised in 1997)
ENV/MC/CHEM(98)17.

EC Directive 99/11/EC of 8 March 1999 (OJ No. L 77/8-21, 23/3/1999)



S. J. Gonsior, M.S. (Date)
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QUALITY ASSURANCE STATEMENT

Compound: HEXABROMOCYCLODODECANE

Title: EVALUATION OF AEROBIC AND ANAEROBIC
TRANSFORMATION OF HEXABROMOCYCLODODECANE IN
AQUATIC SEDIMENT SYSTEMS

This study was examined for conformance with Good Laboratory Practices as published by the USEPA (TSCA), the EC and the OECD. The dates of Quality Assurance activities on this study are listed below.

Study Initiation Date: 20 June 2002

<u>TYPE OF AUDIT:</u>	<u>DATE OF AUDIT:</u>	<u>DATE FINDINGS REPORTED TO STUDY DIRECTOR/MANAGEMENT:</u>
Final protocol	26 June 2002	26 June 2002
Study conduct	10 July 2002	10 July 2002
Protocol, data, and draft report	3 March 2003	4 March 2003

Final report The date of the signature below is the date of the final report audit.

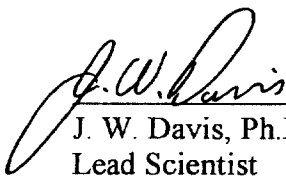
The final report accurately reflects the raw data of the study.

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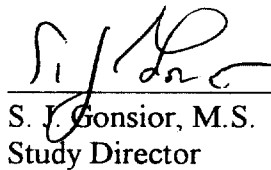
SIGNATURE PAGE

Compound: HEXABROMOCYCLODODECANE

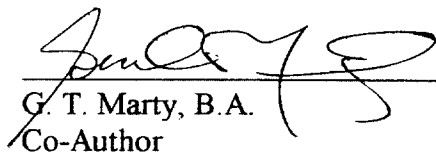
Title: EVALUATION OF AEROBIC AND ANAEROBIC
TRANSFORMATION OF HEXABROMOCYCLODODECANE IN
AQUATIC SEDIMENT SYSTEMS


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Lead Scientist

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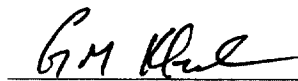

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SUMMARY

The transformation of hexabromocyclododecane (HBCD) was determined in aerobic and anaerobic water/sediment microcosms based on the Organisation for Economic Co-Operation and Development (OECD) Test Guideline 308 "Aerobic and Anaerobic Transformation in Aquatic Sediment Systems." Laboratory batch microcosms were prepared with authentic water and sediment collected from two rivers in the eastern United States. Aerobic microcosms were pre-incubated at 20 ± 1 °C for 49 days and maintained by periodically exchanging the headspace of the microcosms with ambient air to replenish oxygen. Anaerobic microcosms were prepared in an anoxic atmosphere (70% N₂, 28% CO₂, and 2% H₂). The microcosms were pre-incubated at 23 ± 1 °C for 43 to 44 days to allow the microcosms to stabilize. HBCD was then added to the microcosms at nominal concentrations ranging from 34 to 89 ng/g (sediment dry weight). Biologically inhibited (i.e., abiotic) controls were prepared by steam sterilization of the sediment/water mixture prior to the addition of HBCD. Microcosms were incubated in the dark at 20 ± 1 °C for 119 days. The concentration of HBCD in the microcosms was determined at selected time intervals in the water and sediment phases utilizing high performance liquid chromatography-mass spectrometry (LC-MS). Aerobic microcosms were analyzed on days 0, 1, 7, 21, 64, 91, and 119, while anaerobic microcosms were analyzed on days 0, 1, 7, 14, 61(or 62), 91, and 119.

HBCD concentrations decreased over time in both the aerobic and anaerobic microcosms. HBCD concentrations in the viable aerobic microcosms from both river systems decreased at least 90% within 21 days, while the corresponding decreases in the abiotic controls ranged from 7 to 62%. Disappearance of HBCD was observed in both the viable and abiotic anaerobic microcosms with the rate of loss more rapid in the viable microcosms, with HBCD reaching non-detected levels within 7 days. In contrast, HBCD concentrations in the abiotic controls decreased from 48 to 62% after 14 days. Pseudo-first order kinetics rate constants for the biotransformation of HBCD were determined by subtracting the abiotic rate constant from the viable rate constant. Biotransformation half-lives for HBCD in the two river systems were determined to be 11 and 32 days in the

aerobic microcosms and 1.1 and 1.5 days in the anaerobic microcosms. Brominated degradation products were not detected in the sediment and water layers or in the headspace of the microcosms. Based upon these results, HBCD can not be considered "persistent in the environment," since the half-lives in sediments are clearly below the criteria of 120 days as specified in the European Commission's Technical Guidance Document on Risk Assessment (EC 1996).

INTRODUCTION

Hexabromocyclododecane (HBCD) is used as a flame retardant in several applications, including expanded and extruded polystyrene. HBCD has the potential for release into the environment from production processes, and from the processing and disposal of fabricated products containing the compound.

Biodegradation is a major process for removing chemicals from the environment. Aerobic biodegradation processes can predominate in surface waters, surface soils, and the aeration basins of wastewater treatment plants (WWTPs). Anaerobic processes, in contrast, can occur in aquatic sediments, groundwater, and in anaerobic digestion units of WWTPs. Little information is available concerning the aerobic and anaerobic biodegradability of HBCD. The compound exhibited no degradation in the OECD 301D Closed Bottle Test [1]. However, these results may have minimal environmental consequences since the test concentration of HBCD exceeded the reported water solubility of 3.4 µg/L by greater than 2000-fold. The lack of measurable biodegradation in this test was due, in part, to the limited availability of the test material to the microorganisms as well as the low amount of biomass contained in the inoculum.

The objective of this study was to assess the lifetime of HBCD at realistic environmental concentrations in aquatic sediments. The environmental behavior of HBCD is governed by how it partitions between soils, water (sediments) and air. Partitioning and transport of HBCD is dependent upon its intrinsic physical-chemical properties including a low vapor pressure (6.3×10^{-5} Pa), low aqueous solubility (3.4 µg/L), high K_{ow} (Log 5.62) and high soil organic carbon sorption coefficient (Log K_{oc} 4.66) [1]. Based upon these properties there is a high potential for this material to absorb to soil and sediments. In

order to predict the environmental lifetime of a chemical it is important to demonstrate degradation in the environmental compartments where the compound is likely to reside. Since soil and sediments are likely sinks for HBCD it is critical to quantitate the degradation processes in these compartments to better predict the environmental lifetime of HBCD.

The experimental procedure was based on the Organisation for Economic Co-Operation and Development (OECD) Guideline 308 "Aerobic and Anaerobic Transformation in Aquatic Sediment Systems" [2]. Laboratory studies were conducted using microcosms prepared under aerobic and anaerobic conditions. Concentrations of HBCD were determined at selected time intervals in the water and sediment phases using high performance liquid chromatography/mass spectrometry (LC/MS). The analysis allowed the removal of HBCD to be followed, and transformation rates to be determined.

MATERIALS

Test Material

A 25-gram sample of HBCD was obtained from Wildlife International, Ltd., Easton, Maryland on January 8, 2002. The sample was identified as a "Representative Sample of Composite of HBCD – WIL# 5850," with a lot number MC258500102. The sample was a composite of test materials supplied by Albemarle Corporation, Great Lakes Chemical Corporation, and Eurobom B.V. The identity and composition of the sample was characterized by Albemarle Corporation [3]. The identity of the compound was confirmed by Fourier Transform Infrared Spectroscopy. The composition of the test sample, based on the average of triplicate analyses of six separate sub-samples collected from different locations in the bulk sample, was 5.8% alpha isomer, 19.3% beta isomer, and 74.9% gamma isomer. The molecular formula for HBCD is $C_{12}H_{18}Br_6$ ($M_w = 641$).

All other chemicals were obtained from commercial sources. Hexanes (Optima grade) solvent for sample extractions and anhydrous sodium sulfate (Certified ACS) used to dry sediment samples were obtained from Fisher Scientific, Pittsburgh, PA. Laboratory water was purified in a MilliQ® Water Purification System (Millipore Corporation, New Bedford, Massachusetts).

Sediment and Water Samples

Wildlife International (Easton, Maryland) collected aquatic sediments and their associated waters for use in this study on April 23, 2002. Sediments and associated water samples were collected in two freshwater systems (Schuylkill River, Valley Forge, Pennsylvania; Neshaminy Creek, Doylestown, Pennsylvania). Aerobic sediment samples were collected at the surface of the sediment layer (approximately 0 to 5-cm depth). Anaerobic sediment samples were collected in anoxic zones at depths of 3 to 10 cm in the sediment layer. Redox measurements were used to confirm the redox potentials of the sites. Additional measurements obtained during sample collection included temperature and pH. Water and sediment samples were collected separately in glass bottles, packed with ice, and shipped via overnight air express to The Dow Chemical Company in Midland, Michigan. Upon receipt in the laboratory, the sediment and water samples were stored at approximately 4 °C. The sediment samples were passed through 2-mm sieves to remove stones, plant litter, and to improve sample homogeneity. The anaerobic sediments were processed in an anaerobic atmosphere (~70% (vol) N₂, ~28% CO₂, and ~2% H₂) to minimize exposure to oxygen.

Sub-samples of the sediments and water samples were sent to Agvise Labs (Northwood, North Dakota) for characterization. Sediment samples were characterized for texture (sand, silt, clay), pH, organic carbon content, cation exchange capacity, cations (Ca, Mg, Na, K, and H), bulk density, water holding capacity, total nitrogen, phosphorus, soluble salts and microbial biomass. Water samples were analyzed for pH, conductivity, alkalinity, cations (Ca, Mg, Na, K), carbonate, bicarbonate, nitrate, sulfate, chloride, turbidity, and total dissolved solids.

EXPERIMENTAL METHODS

Preparation of Microcosms

Reaction mixtures containing sediment and associated site water from either the Schuylkill River or Neshaminy Creek were prepared in 250-mL serum bottles as described in Table 1. Aerobic microcosms were prepared in ambient air while anaerobic microcosms were prepared in an anaerobic atmosphere. The serum bottles were

described as I-Chem 200- and 300-series (I-Chem Company, New Castle, DE) that were cleaned and certified by the manufacturer to meet U.S. EPA analyte specifications. Microcosms were prepared by combining portions of wet sediment with 100-mL portions of the associated water in the serum bottles. The amounts of sediment added to the microcosms ranged from 14 to 37 grams (dry weight). Sediment moisture content was determined using a standard procedure [4]. The test guidelines recommend water: sediment volume ratios between 3:1 and 4:1 and sediment layer thickness between 1 and 2.5 cm [2]. In this study, the prepared microcosms met the criteria for sediment thickness. However, in order to maintain sufficient headspace in the aerobic microcosms to ensure sufficient oxygen was available, the ratio of water: sediment was reduced to the range of 1.6 to 2.9. The serum bottles were sealed with caps containing Teflon[®]-coated rubber septa.

The sealed microcosms were stored in the dark to recover from the disturbance of sample collection and microcosm preparation, in accordance with guideline recommendations (*i.e.*, stabilization phase). The sealed anaerobic microcosms were stored at 23 ± 1 °C for 43 to 44 days in anaerobic chambers to minimize exposure to oxygen. The aerobic microcosms were stored in ambient air at 20 ± 1 °C for 49 days in a Precision Model 30 MR Low Temperature incubator. The sealed aerobic microcosms were opened at least weekly and the headspace allowed to exchange with the ambient air to ensure that oxygen was present in the headspace. Regular measurements of pH and redox potentials in selected microcosms were used to confirm the stabilization of both aerobic and anaerobic microcosms. Resazurin, a redox dye was added to a subset of anaerobic microcosms at 1 mg/kg to indicate when the redox potential of the reaction mixtures dropped below -110 mV [5]. In addition, methane formation in a subset of anaerobic microcosms was measured to confirm methanogenic conditions. These measurements were supplemented with visual observations of the separation of the water and sediment phases in the microcosms.

Following the stabilization period, the screw-on caps for the aerobic microcosms were removed and the headspace was allowed to exchange with ambient air. The experimental

design for the study is summarized in Table 1. Acetone was used as a carrier solvent to introduce the HBCD into the test microcosms. The HBCD stock solution (5 μ L) was added near the center of the sediment layer, several millimeters below the surface of the sediment. HBCD was added to replicate test mixtures at nominal concentrations ranging from 34 to 89 μ g/kg, based on sediment dry weight, as described in Table 2. These initial HBCD concentrations were chosen to ensure any degradation/removal of HBCD could be followed through at least two half-lives in the sediment phase. Background microcosms, included in the study design to identify possible analytical interferences, received only 5- μ L acetone. Biologically inhibited control mixtures were prepared to evaluate the role that abiotic processes play in the transformation of the test material. Biologically inhibited controls (background and test microcosms) were prepared by steam sterilizing (121 $^{\circ}$ C; 15 psi; 60 minutes) a subset of microcosms on three separate days prior to the addition of the test material. Anaerobic microcosms were amended with HBCD in an anaerobic atmosphere to avoid exposure to oxygen.

Microcosm Incubation

After the addition of HBCD, aerobic and anaerobic microcosms were stored in the dark at 20 ± 1 $^{\circ}$ C in a Precision Model 30 MR Low Temperature incubator for up to 119 days. The concentration of oxygen in the headspace of selected viable aerobic microcosms was monitored to ensure that aerobic conditions were maintained. When oxygen concentrations decreased to the range of 10 to 15%, the sealed aerobic microcosms were opened for at least 15 minutes and the headspace exchanged with ambient air to increase oxygen concentrations in the headspace. The aerobic Schuylkill River microcosms were refreshed after 79 days of incubation, while the aerobic Neshaminy Creek microcosms were refreshed after 21, 51, and 92 days of incubation. Viable microcosms were sacrificed for analysis on at least seven separate sampling days (including day 0). Abiotic controls were analyzed on four separate samplings days (including day 0). The sampling schedules for the different sets of microcosms were adjusted based on HBCD transformation rates.

Microcosms were processed as follows for the analysis of HBCD: Each microcosm was centrifuged for 10 minutes at 1,000 rpm to separate the water and sediment layer (including suspended matter). The water layer was poured into a 125-mL serum bottle (pre-rinsed with hexanes) and mixed on a reciprocating shaker for approximately one day with 30-mL hexanes. The sediment layer was dried with anhydrous sodium sulfate and mixed with 100-mL hexanes on a reciprocating shaker for approximately one day. The Schuylkill River sediments were dried with 75 g of sodium sulfate and the Neshaminy Creek sediments were dried with 100 g of sodium sulfate. Matrix spikes were used to determine the recovery of HBCD from the sediment and water matrices. Matrix spikes were prepared by adding 5 μ L of an HBCD stock solution (250 mg/L in acetone) to duplicate background water and sediment samples prior to the addition of the extraction solvent. The matrix spikes were extracted and analyzed in parallel to the test microcosms in order to determine the recovery of HBCD at each sampling interval. Water and sediment samples from background microcosms (no HBCD added) were extracted and analyzed for potential background analytical interferences on selected days. Method spikes were used to monitor extraction efficiency of HBCD in the assay. Method spikes were prepared by adding 5 μ L of the HBCD stock solution to 100 mL of water and mixing on a reciprocating shaker for approximately one day with 30-mL hexanes. The hexanes extracts were washed with water to remove salts, evaporated to dryness, and re-dissolved in acidified HPLC mobile phase. The re-constituted samples were analyzed for HBCD using LC-MS. Details of the sample preparations are included in Appendix A.

Aqueous bromide levels were measured in selected aerobic microcosms at the conclusion of the study to determine if bromide was released from the HBCD added. The microcosms were vigorously mixed for several minutes, then allowed to settle overnight. The purpose of the mixing was to uniformly distribute any bromide ions in the aqueous layer. The aqueous layer was centrifuged to remove solids and the clarified supernatant liquid layer was analyzed for bromide by ion chromatography.

At the conclusion of the study, the headspace of selected blank and test microcosms was passed through SKC Anasorb CSC (Coconut Shell Charcoal) carbon tubes to collect

possible volatile brominated degradation products. One end of an adsorbent carbon tube was connected to a 60-mL plastic syringe and the other end was connected to a syringe needle inserted into the septum of the microcosm. A second needle was inserted through the septum with the tip positioned above the water layer. Approximately 300 mL of ambient air (5 x 60-mL portions) was drawn through the microcosm headspace, effectively flushing the headspace of the microcosm through the carbon adsorbent. The carbon adsorbent was extracted with a 1.3 mL of acetonitrile for at least 16 hours. The extract was mixed with 0.2 mL of acidified water and analyzed for brominated degradation products by HPLC-mass spectrometry and GC-mass spectrometry.

The headspace of five microcosms (single viable background and duplicate test; single abiotic background and test) were collected and analyzed from each river system for both aerobic and anaerobic conditions. The microcosms had been sealed for the entire study with the following exception. The viable Neshaminy Creek microcosms were opened in ambient air after 51 days of incubation to replenish depleted oxygen levels in the headspace. These microcosms were sealed for the remainder of the study (68 days of additional incubation) to allow possible volatile degradation products to accumulate in the microcosm headspace.

Analytical Methods

A high performance liquid chromatography/mass spectrometry (LC/MS) method was used to measure HBCD concentrations in this study. This assay was derived from a method previously used for the analysis of HBCD in water and fish tissues [6,7]. Extensive method development was required to identify sample extraction and instrument parameters that would provide a sensitive, reproducible assay for the environmentally realistic concentrations of HBCD used in this study. Initial method development was performed on a PE Sciex API 2000 LC/MS/MS using atmospheric pressure chemical ionization (APCI). Hexanes, toluene, isooctane, and methylene chloride gave comparable extraction efficiencies for HBCD in environmental matrices. Hexanes was chosen as the extraction solvent for the HBCD assay because its low density (*i.e.*, floats on water) and volatility were ideally suited to recover and concentrate HBCD residues

from the microcosms used in this study. Additional recovery experiments comparing hexanes and methylene chloride/sonication extraction of sediment samples confirmed the suitability of hexanes solvent for the assay. Due to instrument problems, the PE Sciex API 2000 LC/MS/MS system was replaced with an Agilent Technologies LC-MS with atmospheric pressure photoionization (APPI) prior to the start of the HBCD transformation studies.

Sample work-up prior to LC-MS analysis consisted of the following: Hexanes extracts of water and sediment samples were blown to dryness and reconstituted in acetonitrile/water (85/15) containing 0.1% HCl. Extracts of the sediment samples were washed with water to remove salts prior to the evaporation step. The samples were analyzed by LC-MS with APPI. Instrument conditions are included in Appendix B. The transformation of HBCD was followed by monitoring the HBCD molecular ion ($m/z = 641$ amu) and its bromine fragment ion ($m/z = 79$ amu). Representative spectra are shown in Figure 1. By monitoring the bromine fragment ion, the analysis also provided the potential to detect brominated degradation products. Concentrations of HBCD were quantified based on the HBCD molecular ion ($m/z = 641$ amu). The three isomers present in the test material (alpha, beta, and gamma) could be resolved in the LC-MS assay. However, only the major gamma isomer (75% of HBCD) was used to quantify HBCD concentrations in the microcosms (the alpha and beta isomers would have been detected had they been present at levels similar to the starting gamma concentration). Calibration curves were generated at the beginning and end of each sample set to confirm proper operation of the instrumentation and linearity of detector response. To compensate for any change in response of the LC-MS, a reference standard was repeatedly analyzed throughout the sample set (*e.g.*, every fourth or fifth analysis). Response factors were determined for the reference standard and an average response factor was used to calculate HBCD concentrations for samples bracketed by the reference standards. Measured HBCD concentrations were corrected for recovery based on matrix spikes prepared for each sample set.

Method spikes (water spiked with HBCD) were included in each sample set to confirm the reliability of the extraction procedure. The average recovery of HBCD in the method

spikes prepared over a 127 day period was $93 \pm 13\%$ (Appendix C). HBCD was stable in stock solutions prepared in acetone that were used to dose the test microcosms and prepared calibration standards. The HBCD stock solution used to spike the test microcosms was re-assayed with a fresh stock solution prepared 106 days later. The results showed 109% of the original concentration of HBCD present, confirming the stability of the HBCD stock solution.

Though authentic standards of possible HBCD degradation products were not commercially available, four brominated aliphatic compounds were evaluated in the LC-MS assay to assess the capability to detect brominated compounds. The four compounds, hexabromocyclohexane, 1,2-dibromocyclooctane, trans 1,2-dibromocyclohexane, and 1,10-dibromodecane, each showed a response in the assay at 79 m/z. Thus, the LC-MS assay was capable of detecting a range of brominated compounds indicating that this analysis had the potential for detecting brominated products which could typically be produced from HBCD degradation.

The acetonitrile extracts from the carbon adsorbent tubes used to collect possible volatile brominated degradation products of HBCD in the microcosm headspace gases were analyzed by LC-MS and GC-MS without further preparation. The bromine fragment ion ($m/z = 79$ amu) was monitored to detect possible brominated degradation products. Instrument conditions for the GC-MS assay are included in Appendix D.

Bromide concentrations in selected aqueous samples were determined using ion chromatography. Instrument conditions are listed in Appendix E.

Methane concentrations in the headspace of selected anaerobic microcosms were measured by GC with flame ionization detection (FID). Instrument conditions are listed in Appendix F. Methane gas standards were prepared by diluting a gas standard containing 10% (mole %) methane, ethane, and ethylene (BOC Gases, Port Allen, Louisiana) with air in septum-capped headspace vials. Methane concentrations in the headspace of selected microcosms was determined using an external standard calculation.

Oxygen concentrations in the headspace of microcosms were measured using an S2000 Fiber Optic Spectrometer (Ocean Optics, Inc., Dunedin, Florida). The fiber optic sensor

was calibrated daily using laboratory air (20.95% oxygen) and argon gas (0% oxygen) contained in a sealed serum bottle.

The pH of aqueous samples was determined using a standard procedure [8]. An Orion Sure-Flow Ross Semi-Micro Electrode was connected to an Orion model 920 pH meter. The pH probe was calibrated with standard buffered solutions at pH 4, 7, and 10 (Fisher Scientific).

The oxidation-reduction potential (Eh) of the aqueous layer of selected microcosms was measured using an Accumet probe connected to an Orion model 920 meter. Zobell's solution ($3 \times 10^{-3}M$ potassium ferrocyanide and $3 \times 10^{-3}M$ potassium ferricyanide in 0.01M KCl) was prepared to calibrate the probe. The Eh value was determined by correcting the E_{observed} value. The correction was based on the measured Eh value for the Zobell's solution and temperature [9].

Temperature measurements in the anaerobic chambers were made with ASTM 63 thermometers [10]. Temperature measurements in the incubators were made using an automated laboratory system [11].

Data Analysis

Kinetics of HBCD loss were determined by plotting the natural logarithm of the HBCD concentration as a function of time. Pseudo-first order rate constants (k) were determined by regression analysis. Half-lives for HBCD losses were calculated using the relationship $t_{1/2} = \ln 2/k$. Loss of HBCD due to biological processes (*i.e.*, biotransformation) was determined by subtracting the rate of loss measured in abiotic controls from the rate of loss measured in viable reaction mixtures.

RESULTS AND DISCUSSION

River Water and Sediment Characterizations

Water quality parameters measured during the collection of water samples are summarized in Table 3. The pH of the water samples at the four sites ranged from 7.0 to 8.7, while dissolved oxygen measurements ranged from 8.1 to 12.4 mg/L. Note that the water samples collected at each of the sites were aerobic, even if the collected sediment was anaerobic. The water temperature at the sites ranged from 12 to 14 °C. The redox potentials measured for the aerobic sediments ranged from -18 to 151 mV, while the redox potentials for the anaerobic sediments ranged from -199 to -207 mV.

Characterization of the sediment samples showed a diverse range of properties (Table 4). The classifications for the four sediment samples ranged from sand to a sandy loam. The percent organic carbon ranged from 0.4 to 4.2, and microbial biomass ranged from 111 to 547 µg/g using an extraction method [12].

The water quality parameters of the water samples are summarized in Table 5. The pH of the water samples ranged from 6.5 to 8.0. The remaining parameters for the four sets of water samples were similar. Conductivity ranged from 0.38 to 0.64 mmhos/cm and alkalinity ranged from 58 to 83 mg CaCO₃/L. Total dissolved solids ranged from 190 to 316 ppm.

Methane and Redox Monitoring.

Microcosms were incubated for periods ranging from 43 to 49 days prior to the addition of HBCD. The headspace of selected anaerobic microcosms was monitored for the presence of methane during the stabilization phase (Table 6). Methane was detected in both the Schuylkill River and Neshaminy Creek anaerobic microcosms during the stabilization phase. Methane concentrations reached 2.5% after 36 days in the Schuylkill River microcosms and 10.4% after 35 days in the Neshaminy Creek microcosms. The formation of methane, together with low redox conditions, demonstrated that anaerobic conditions were established in the sediment microcosms during the stabilization phase.

The redox potentials of the water layers immediately above the sediment layers were regularly monitored in selected microcosms. For the anaerobic microcosms, the redox potentials of the water layers decreased during the stabilization period (Table 7). The redox potentials in the anaerobic Schuylkill microcosms decreased from 151 ± 77 mV after 8 days of incubation, to 69 ± 6 mV after 44 days. Following the addition of HBCD, the redox potentials of the water layers in the microcosms continued to decrease, falling to -75 ± 35 mV for background microcosms and -320 ± 42 mV for test microcosms after 119 days. These results confirmed that anaerobic conditions were maintained in this set of microcosms. The redox potential for one abiotic control was -400 mV.

For the anaerobic Neshaminy Creek microcosms during the stabilization period, the measured redox potentials of the water layers decreased from 90 ± 16 mV after 7 days to 66 ± 5 mV after 43 days of incubation (Table 7). Following the addition of HBCD, the redox potentials of the water layers in the microcosms continued to decrease. At the end of the study (119 days), the redox potentials were -424 ± 6 mV for background microcosms and -410 ± 0 mV for test microcosms, confirming that anaerobic conditions were maintained. The redox potential for one abiotic control was -402 mV.

Rezasurin dye was added as a redox indicator to selected anaerobic microcosms during their preparation. This indicator changes from pink to colorless when the redox potential falls below -110 mV [5]. The redox indicator changed to colorless within six days in the anaerobic Neshaminy Creek microcosms and within 20 days in the anaerobic Schuylkill River microcosms. These results indicate that the redox potentials of the anaerobic microcosms were decreasing during the stabilization phase. The redox indicators remained colorless throughout the 119-day incubations of the microcosms following HBCD addition.

During the stabilization phase the redox potentials for the aerobic Schuylkill microcosms increased from 220 ± 100 mV after 14 days of incubation to 344 ± 26 mV at the end of the 49-day stabilization phase (Table 8). Following the addition of HBCD, the redox potential of the microcosms increased to 423 ± 11 mV at the conclusion of the study (119 days). The redox potential of the abiotic controls was 357 ± 5 mV at the end of the study.

Little change was observed in redox potentials in the aerobic Neshaminy Creek microcosms during the stabilization phase, with readings of 69 ± 14 mV after 14 days and 70 ± 23 mV after 49 days in the water layer (Table 8). At the conclusion of the study, the redox potentials had increased to 142 ± 10 mV and 139 ± 18 mV for background and test microcosms, respectively. These results indicate that aerobic conditions were maintained in the study. The redox potentials for the abiotic controls at the end of the study were 112 ± 34 mV.

pH and Oxygen Monitoring

The pH of the water layers in the four sets of microcosms showed little change (0.7 pH units or less) during this stabilization phase (Tables 9 and 10). During the test phase, the pH of the water layers of the aerobic microcosms ranged from pH 7.0 to 7.4 in the Schuylkill River microcosms and pH 6.7 to 7.2 in the Neshaminy Creek microcosms. For the anaerobic microcosms, the pH of the water layers ranged from 6.5 to 6.7 for the Schuylkill River microcosms and pH 6.4 to 6.6 for the Neshaminy Creek microcosms.

Oxygen concentrations were routinely monitored in the headspace of the aerobic microcosms following the addition of HBCD (Table 11). The headspace of the viable aerobic Schuylkill River microcosms was exchanged with ambient air after 79 days of incubation to replenish oxygen depleted from biological activity. Similarly, the headspace of the viable aerobic Neshaminy Creek microcosms was exchanged with ambient air after 21, 51, and 92 days of incubation to replenish reduced oxygen levels. Note that oxygen concentrations in two abiotic controls from the Neshaminy Creek microcosms decreased to approximately 5%, suggesting that the heat treatment did not completely inhibit biological activity in these microcosms.

Following preparation of the microcosms, the sediment settled from the water layer within several days. Table 12 summarizes measurements of the thickness of the water and sediment layers following 14 and 119 days of incubation during the test phase. The water layers of the microcosms ranged from 3.5 to 4.1 cm, while the sediment layers ranged from 1.4 to 2.2 cm. The ratios of the thickness of the water to sediment layers

ranged from 1.6 to 2.9. Changes in the thickness of the water and sediment layers did not exceed 13% during the study, confirming the stabilization of the microcosms.

Lifetime of HBCD in Water/Sediment Microcosms

HBCD concentrations measured in the water and sediment layers of each microcosm were combined and reported on the basis of the dry sediment weight of the microcosm. An interfering peak was observed in LC-MS chromatograms when organic extracts of the Neshaminy Creek microcosms were analyzed for HBCD. This interfering peak was present in background microcosms that were not amended with HBCD. The identity of the interfering compound was not specifically determined, but it did produce an ion at 641 amu and bromine consistent response at 79 amu. HBCD concentrations were corrected for the background interference. The average concentration of this interfering compound (based on HBCD response) in the viable aerobic microcosms was 19.5 ± 3.0 ng/g (days 0, 7, and 119). Day 0 value for this interference in the abiotic controls was 5.2 ng/g. In the anaerobic Neshaminy Creek microcosms, the corresponding values for the interfering compound were 24.7 ± 1.3 ng/g in the viable microcosms (days 0, 1, and 119), and 4.9 ng/g in the abiotic control (day 0). The lower concentrations present in the abiotic controls suggest that the interfering compound may have undergone degradation during the heat treatment used to prepare the abiotic microcosms.

Little HBCD was detected in the water layers of the microcosms. These results are consistent with the low water solubility of HBCD and high partition (*e.g.*, octanol/water) coefficients [1]. HBCD concentrations decreased over time in both viable and abiotic microcosms under both aerobic and anaerobic conditions. In the aerobic Schuylkill River microcosms, HBCD concentrations were reduced from 31.9 ng/g (based on dry weight of sediment) to non-detected levels within 64 days in the viable microcosms (Table 13 and Figure 2). Over the same time period, HBCD concentrations were reduced 31% in the abiotic controls, from 31.3 to 21.6 ng/g. Note that the measured day 0 concentrations were within 92% of the 34 ng/g nominal HBCD concentration added to the microcosms. This good agreement between measured and nominal HBCD values confirmed the suitability of the LC-MS assay.

Figures 3 through 6 include LC-MS chromatograms that illustrate the loss of HBCD in the aerobic Schuylkill River Microcosms. The upper chromatogram in each figure shows the molecular ion of HBCD (641 m/z) while the lower chromatogram shows the bromine fragment (79 m/z). Note the decrease in both peaks over time for day 0 (Figure 3) day 21 (Figure 4) and day 64 (Figure 5) viable microcosms. In contrast, HBCD peaks are still observed in the abiotic control on day 64 (Figure 6). Note that the alpha and beta isomers of HBCD were not detected in viable microcosms after day 0. These results indicate that the major gamma isomer of HBCD did not convert to the alpha and beta isomers in this test system. Also, note that there were no detectable bromine fragment ions at retention times other than the HBCD retention time (*i.e.*, no brominated degradation products were detected).

HBCD concentrations in the viable aerobic Neshaminy Creek microcosms decreased 93% in 21 days (80.7 to 5.7 ng/g) and 62% in the abiotic controls (58.3 to 22.0 ng/g) during the same period (Table 14 and Figure 7). HBCD concentrations on day 0 ranged from 97 to 135% of the nominal 60 ng/g of HBCD added to the microcosms.

LC-MS chromatograms for day 0 (Figure 8) and day 91 (Figure 9) illustrate the loss of HBCD in the viable aerobic microcosms prepared from Neshaminy Creek sediment. Note in Fig 9A the presence of an interfering peak in the LC-MS chromatogram at retention times (RT) similar to that observed for HBCD. This interfering peak was present in the Neshaminy Creek chromatograms prior to the addition of HBCD to the sediment (Figure 10- viable background microcosms). This interfering peak was present in the Neshaminy Creek chromatograms throughout the study. Conversion of the major gamma isomer of HBCD to the minor isomers was not observed.

Rapid disappearance of HBCD was observed in the anaerobic Schuylkill River microcosms. In the viable microcosms, HBCD concentrations decreased from 27.7 ng/g on day 0 to non-detected levels within seven days (Table 15 and Figure 11). For the abiotic controls, HBCD concentrations decreased from 27.2 to 14.2 ng/g over 14 days (48% decrease). At the next sampling point (61 days), HBCD was non-detected in the abiotic microcosms. Note that for both viable and abiotic controls, the measured day 0

values were approximately 44% of the nominal 63 ng/g of HBCD added to the microcosms. The reason for the lower recovery of HBCD from these reaction mixtures is not known, but the results suggest a rapid removal mechanism was operative both biotically and abiotically. HBCD disappeared more rapidly in the biologically active microcosms compared to the abiotic controls, indicating that both biotic and abiotic mechanisms may be responsible.

LC-MS chromatograms for day 0 (Figure 12) and day 14 (Figure 13) illustrate the loss of HBCD in the viable anaerobic microcosms prepared from Schuylkill River sediment. Conversion of the major gamma isomer of HBCD to the minor isomers was not observed. HBCD concentrations in the viable anaerobic Neshaminy Creek microcosms decreased from 39.1 ng/g to non-detected levels after 7 days (Table 16 and Figure 14). In contrast, HBCD concentrations in the abiotic controls decreased 62% in 14 days and were non-detected at 62 days. Measured HBCD concentrations on Day 0 were approximately 43% of the nominal 89 ng/g of HBCD added to the microcosms.

LC-MS chromatograms for day 0 (Figure 15) and day 119 (Figure 16) illustrate the loss of HBCD in the viable anaerobic microcosms prepared from Neshaminy Creek sediment. Again, note the interfering peak in the LC-MS chromatogram for the viable background microcosm on day 0 (Figure 17) that was present throughout the study. Conversion of the major gamma isomer of HBCD to the minor isomers was not observed.

Kinetics of HBCD Removal

Degradation rates of chemicals are routinely approximated by using kinetics that are first order with respect to substrate concentration. This approach is commonly used when chemicals are present at low concentrations in the environment (low $\mu\text{g/L}$ or $\mu\text{g/kg}$). Pseudo-first order kinetics can be assumed when there are negligible changes in biomass levels that occur as a result of growth [13]. In this study pseudo-first order rate constants for HBCD removal in the sediment microcosms were determined from plots of the natural logarithm of the HBCD concentration versus time (Appendices G and H). Results of the kinetic analysis are summarized in Table 17. Pseudo-first order biotransformation rate constants were determined by subtracting the abiotic rate constant from the viable

rate constant. Based on these rate constants, the biotransformation half-lives for HBCD under aerobic conditions were 11 and 32 days for the Schuylkill River and Neshaminy Creek microcosms, respectively. The half-lives for the biotransformation of HBCD under anaerobic conditions were 1.5 and 1.1 days for the Schuylkill River and Neshaminy Creek microcosms, respectively. Note that the biologically mediated transformation rates were up to 18 times faster than the abiotic rates in the microcosms.

HBCD Degradation Products

HBCD degradation products were not detected in extracts of the sediment and water layers. The LC-MS assay routinely monitored the HBCD molecular ion at 641 amu and the bromine 79 amu fragment ion in each sample analyzed. Degradation products containing bromine would be expected to produce a bromine fragment ion and thus be detected in the assay.

The headspace of selected microcosms was passed through carbon adsorbent tubes at the conclusion of the study to collect possible volatile brominated degradation products from HBCD. Organic extracts of the carbon tubes were analyzed by LC-MS and GC-MS. No brominated compounds were detected in the assays. Bromide ion concentration was determined in the aqueous phase of selected microcosms at the end of the study. The purpose was to determine if the loss of HBCD observed in the microcosms resulted in the release of bromide ion. Complete degradation of the 1,250 ng of HBCD in the microcosms would have resulted in the release of 938 ng of bromide (HBCD contains 75% bromine by weight). Bromide levels in aerobic microcosms after 119 days of incubation were as follows:

Microcosm	Nominal concentration of Bromine added as HBCD (ng/L) x 10 ⁻⁶	Bromide concentration in aqueous phase (ng/L) x 10 ⁻⁶
Schuylkill River Background	–	2.04
Schuylkill River Test	0.0083	1.92
Neshaminy Creek Background	–	1.17
Neshaminy Creek Test	0.0076	1.19

The background levels of bromide present in the blank microcosms were 150- to 250-fold higher than the theoretical concentration that could be released from the amount of HBCD added. These background levels were too high to permit quantitation of bromide released from the HBCD.

DISCUSSION

The purpose of this study was to determine the environmental lifetime of HBCD under realistic environmental conditions. Laboratory microcosms were used to evaluate the transformation of HBCD in aerobic and anaerobic water/sediment microcosms. Sediment degradation processes are expected to play a major role in determining the environmental lifetime of HBCD since, upon release into the environment, the majority of HBCD is likely to partition into the soil or sediment compartments. In this study, HBCD loss was observed in both viable and abiotic sediments although the rates were appreciable faster in the viable sediments. Biologically mediated transformation processes (*i.e.*, biotransformation) accelerated the rate of loss of HBCD when compared to the biologically inhibited (*i.e.*, heat-treated) microcosms. Brominated degradation products were not detected in any of the sediment microcosms.

Over the last several years a number of international protocols (EC 1996, UNECE 1998, UNEP 2000) have been put forth for the classification of chemicals as persistent (P), bioaccumulative (B), and toxic [14, 15, 16]. The criteria for persistence in these initiatives includes half-lives in soil and sediments ranging from 120 to 180 days. In this investigation the resulting biotransformation half-lives for HBCD in the two river systems were determined to be 11 and 32 days in the aerobic sediments and 1.1 and 1.5 days in the anaerobic sediments, respectively. Based upon these results HBCD can not be considered "persistent in the environment" since the half-lives in soil are clearly below the criteria specified by the various international protocols and specifically below the 120 days value specified in the European Commission's Technical Guidance Document on Risk Assessment (EC 1996).

Limited information is available for the reactions of HBCD in the environment. However, in a separate soil degradation study conducted by Lickly and coworkers [17]

they observed the aerobic degradation and mineralization of a similar type of cyclic aliphatic halogenated fire retardant, FR-651A (mixture of pentabromo-chlorocyclohexane, tetrabromo-dichlorocyclohexane, and tribromotrichlorocyclohexane). They reported a soil half-life of ~11 days based upon disappearance of ^{14}C -FR-651A from soil. Complete degradation of ^{14}C -FR-651A was also observed with a mineralization half-life on the order of 93 days.

An examination of the reactions of brominated aliphatic compounds that contain structural features similar to HBCD can also provide insight into the possible reaction pathways available for HBCD. Brominated aliphatic compounds are known to be susceptible to both hydrolytic and nucleophilic attack. The reactivity of halogenated aliphatic compounds depends of the strength of the carbon-halogen bond, and increases in the order of $\text{F} < \text{Cl} < \text{Br}$. For example, the hydrolysis half-lives at pH 7 and 25 °C for methyl fluoride, methyl chloride, and methyl bromide are 1.1×10^4 , 340, and 20 days, respectively [18]. At environmental pH's neutral and base catalyzed hydrolysis reactions are most important, and depend on the degree and patterns of halogen substitution [18].

Sulfur based nucleophiles can react rapidly with halogenated aliphatic compounds, thereby reducing their lifetimes in the environment. The half-life of 1-bromohexane in water at 25 °C was reduced from 20 days to approximately one day in 5 mM HS^- or 0.07 mM polysulfide (S_x^{2-}) [19]. Similarly, the half-life of 1,2-dibromoethane in water at 25 °C was reduced from 1,000 days to 4 days in the presence of 5 mM HS^- . Sulfide and polysulfides would be expected to be present in sediments at low redox potentials. Thus, reaction of HBCD with these sulfur-based nucleophiles may partly explain the loss of HBCD in the microcosm studies.

Since HBCD contains three pairs of vicinal bromine atoms, simple aliphatic compounds containing vicinal bromine atoms can provide insight into possible reaction pathways. For example, 1,2-dibromoethane reacts with nucleophiles via both substitution and elimination reactions [18]. Reaction with HS^- via an "SN2" substitution reaction results in the formation $\text{HS-CH}_2\text{-CH}_2\text{-SH}$, while an elimination reaction results in the formation of $\text{H}_2\text{C} = \text{CHBr}$. A combination of elimination and substitution reactions can result in

the formation of a mixture of $\text{HO-CH}_2\text{-CH}_2\text{-OH}$ and $\text{H}_2\text{C} = \text{CHBr}$. Similar types of reactions may have occurred in this study. Note that the water from both the Schuylkill and Neshaminy Rivers contained sulfate at levels > 40 ppm (Table 5). There is a high likelihood that sulfide like compounds were generated in the sediment microcosms, as these mixtures became anoxic.

Halogenated aliphatic compounds are susceptible to reductive dehalogenation reactions in natural reducing environments. The rates of reactions vary depending on the strength and electron affinity of the carbon-halogen bond, and the stability of the carbon-radical species resulting from the initial electron transfer that occurs [20]. Vicinal dehalogenation reactions are particularly important, and are dependent on the halogen atom. Half-lives for vicinal dehalogenation of 1,2-diiodoethane, 1,2-dibromoethane, and 1,2-dichloroethane are 0.6, 55, and 1.8×10^4 hours, respectively [20]. The rapid disappearance of HBCD in the anaerobic sediment microcosms may be partly explained by reductive dehalogenation reactions. In addition, the disappearance of HBCD in the aerobic sediment microcosms may also be at least partly explained by reductive dehalogenation reactions. Anaerobic gradients often occur below the surface of sediments that are exposed to an aerobic water column [2]. Such gradients would be expected to form in the static microcosms used in this study.

QUALITY ASSURANCE

This study was conducted in accordance with Good Laboratory Practice Standards [21, 22, 23, and 24]. The study conduct and data generated were reviewed according to the procedures of the Quality Assurance Unit of Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan.

ARCHIVING

The data, protocol, protocol changes/revisions, and final report are archived at Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan.

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Table 1. Description of Reaction Mixtures for Aerobic and Anaerobic Transformation Experiments in Aquatic Sediments

Reaction Mixture	Description	Analysis	Purpose
Viable Test	Sediment/water mixtures + HBCD (in carrier solvent)	LC/MS	Evaluate role of biological processes in the transformation of HBCD in water and sediment
Viable Background	Sediment/water mixtures + carrier solvent	LC/MS	Evaluate possible analytical interferences of sample matrix
Abiotic Test	Heat sterilized sediment/water mixtures + HBCD (in carrier solvent)	LC/MS	Evaluate role of abiotic processes in the transformation of HBCD in water and sediment
Abiotic Background	Heat sterilized sediment/water mixtures + carrier solvent	LC/MS	Evaluate possible analytical interferences of sample matrix

Note: 1) HBCD was added to sediment mixtures at concentrations ranging from 34 to 89 ng/g based on the sediment dry weight
2) Acetone was used as carrier solvent

Table 2. Preparation of Microcosms

Microcosm Description	Moisture Content of Sediment (%)	Dry Weight of Sediment Added (g)	Nominal HBCD Addition (ng/g dry weight)
Aerobic Schuylkill River	26	37	34
Aerobic Neshaminy Creek	53	21	60
Anaerobic Schuylkill River	48	20	63
Anaerobic Neshaminy Creek	61	14	89

Table 3. *In Situ* Measurements of Water and Sediment at Collection Sites

Water Samples

Sample	Description	Temperature (°C)	pH	Dissolved Oxygen (mg/L)
Schuylkill River	Site A	14	7.0	8.4
Schuylkill River	Site B	12	7.1	8.1
Neshaminy Creek	Site A	14	8.6	12.4
Neshaminy Creek	Site B	13	8.7	12.0

Site A – Water collected above aerobic sediment site

Site B – Water collected above anaerobic sediment site

Sediment Samples

Sample	Description	Collection Depth (cm)	Redox Potential (mV)
Schuylkill River	Site A - Aerobic	0-5 cm	151
Schuylkill River	Site B - Anaerobic	3-10 cm	-207
Neshaminy Creek	Site A - Aerobic	0-5 cm	-18
Neshaminy Creek	Site B - Anaerobic	5-10 cm	-199

Table 4. Sediment Characterization

Sample	Description	% Organic Carbon*	Classification (USDA)	% Sand	% Silt	% Clay	Microbial Biomass $\mu\text{g/g}$
Schuylkill River	Aerobic	0.4	Sand	95	4	1	111
Schuylkill River	Anaerobic	2.5	Loamy Sand	87	6	7	125
Neshaminy Creek	Aerobic	4.2	Sandy Loam	65	20	15	547
Neshaminy Creek	Anaerobic	4.2	Sandy Loam	74	20	6	218

*calculated from percent organic matter values determined from Walkley Black method

calculation: $(\% \text{ organic matter}) \times 0.58 = \% \text{ organic carbon}$

Table 5. Characterization of Water

River	Schuylkill River		Neshaminy Creek	
	<i>Aerobic</i>	<i>Anaerobic</i>	<i>Aerobic</i>	<i>Anaerobic</i>
pH	7.7	6.5	8.0	6.7
Conductivity (mmhos/cm)	0.39	0.38	0.64	0.62
Alkalinity (mg CaCO ₃ /L)	59	58	83	80
Turbidity (NTU)	7.1	3.3	1.9	3.0
Total dissolved solids (ppm)	190	194	308	316
Ca (ppm)	27	27	33	33
Mg (ppm)	10	10	12	12
Na (ppm)	25	25	60	59
K (ppm)	4	4	7	7
Carbonate (meq/L)	nd	nd	nd	nd
Bicarbonate (meq/L)	1.2	1.2	1.6	1.5
Nitrate (ppm)	2.2	2.2	3.7	3.7
Sulfate (ppm)	41	42	44	44
Chloride (ppm)	40	40	90	92

Table 6. Headspace Methane Concentrations in Selected Anaerobic Microcosms during Stabilization Phase

(% vol/vol)					
Incubation Time (days):	16	23	31	36	
Schuylkill River	0.2 ± 0.3 ^a	0.1 ± 0.1	1.2 ± 0.1	2.5 ± 0.8	
	n = 5	n = 3	n = 3	n = 3	
Incubation Time (days):	15	22	30	35	
Neshaminy Creek	7.1 ± 4.1	6.3 ± 4.2	8.4 ± 1.9	10.4 ± 0.9	
	n = 5	n = 3	n = 3	n = 3	

^a ± 1 Standard deviation

Table 7. Redox of Water Layer in Selected Anaerobic Microcosms

		Eh (mV)					
Incubation Time (days):	8^a	16^a	23^a	30^a	36^a	44^a	119^b
Viable Schuylkill River Background	151 ± 77 ^c	143 ± 21	66 ± 5	59 ± 1	75 ± 6	69 ± 6	-75 ± 35 -320 ± 42 (-400) ^d
Incubation Time (days):	7	15	22	29	35	43	119
Viable Neshaminy Creek Background	90 ± 16	60 ± 3	66 ± 6	46 ± 7	63 ± 2	66 ± 5	-424 ± 6 -410 ± 0 (-402) ^d

^aStabilization time following preparation of microcosms prior to HBCD addition

^bIncubation time following HBCD addition

^c± 1 Standard deviation

^dRedox potential for abiotic control

Table 8. Redox of Water Layer in Selected Aerobic Microcosms

Incubation Time (days):	14 ^a	22 ^a	29 ^a	36 ^a	42 ^a	49 ^a	91 ^b	119 ^b
Schuylkill River Background	220 ± 100 ^c	267 ± 107	290 ± 59	365 ± 68	366 ± 16	344 ± 26	483 ± 0	423 ± 11 (357 ± 5) ^d
Neshaminy Creek Background	69 ± 14	56 ± 10	49 ± 9	117 ± 54	71 ± 37	70 ± 23	— ^e	142 ± 10 139 ± 18 (112 ± 34) ^d

^aStabilization time following preparation of microcosms prior to HBCD addition

^bIncubation time following HBCD addition

^c± 1 Standard deviation

^dRedox potential for abiotic control

^eNo measurement made

Table 9. pH of Water Layer for Selected Aerobic Microcosms

Incubation Time (days):	14 ^a	22 ^a	29 ^a	36 ^a	42 ^a	49 ^a	91 ^b	119 ^b
Schuylkill River Background	7.2 7.2	7.6 7.3	7.7 7.5	7.9 7.7	7.9 7.9	7.5 7.6	7.4 7.0	7.2 (7.1) ^c 7.0 (7.3) 7.0
Schuylkill River Test	— ^d	—	—	—	—	—	7.2 7.4	—
Neshaminy Creek Background	7.0 7.1	7.2 7.2	7.2 7.0	7.2 7.3	7.3 7.1	7.2 7.0	6.8 6.7 7.2	6.8 (7.0) 6.8 (6.7) 7.0
Neshaminy Creek Test	—	—	—	—	—	—	6.9	7.1 6.9

^aStabilization time following preparation of microcosms prior to HBCD addition

^bIncubation time following HBCD addition

^c() indicates value for abiotic control

^dNo measurement made

Table 10. pH of Water Layer for Selected Anaerobic Microcosms

Incubation Time (days):	8 ^a	16 ^a	23 ^a	30 ^a	36 ^a	44 ^a	91 ^b	119 ^b
Viable Schuylkill River Background	6.5 6.5	6.3 6.4	6.4 6.4	6.5 6.5	6.5 6.5	6.5 6.5	6.5 6.5	6.6 (6.6) ^c 6.6
Viable Schuylkill River Test	— ^d	—	—	—	—	—	—	6.7 6.6

Incubation Time (days):	7 ^a	15 ^a	22 ^a	29 ^a	35 ^a	43 ^a	91 ^b	118 ^b
Viable Neshaminy Creek Background	6.4 6.4	6.4 6.4	6.4 6.4	6.4 6.5	6.4 6.5	6.4 6.5	6.5 6.5	6.4 (6.5) 6.6 6.5
Viable Neshaminy Creek Test	—	—	—	—	—	—	—	6.6

^aStabilization time following preparation of microcosms prior to HBCD addition

^bIncubation time following HBCD addition

^cpH value for abiotic control

^dNo measurement made

Table 11. Oxygen Concentrations in the Headspace of Selected Aerobic Microcosms

Schuykill River Microcosms **Percent Oxygen**

Incubation Time (days):	7	13	20	21	28	35	43	47	64	72	79	91	92	100	114	119
Viable Background	19 21	21 21	19 18	— ^a	21 20	21 21	21 21	22 21 18 22	19 19	17 19	21 15	20 18	20	19 17	20 17	20 21 20 18
Viable Test	21 21	—	19 19	—	—	—	—	22 13 22 21	19 17	—	20 12 21 11	19 20	—	—	—	21 13
Abiotic Background	—	—	—	18 20	—	—	—	—	12 17	—	—	—	—	—	—	16 19
Abiotic Test	—	—	—	19 18	—	—	—	—	17 16	—	—	—	—	—	—	15 12

Note: Headspace of viable microcosms exchanged with ambient air after 79 days of incubation.

^aNo measurement made

Table 11. Oxygen Concentrations in the Headspace of Selected Aerobic Microcosms (continued)
Neshaminy Creek Microcosms

Percent Oxygen

Incubation Time (days):	7	13	20	21	28	35	43	51	64	72	79	91	92	100	114	119
Viable Background	16	13	10	— ^a	16	12	18	13	18	19	20	11	16	7	17	18
	16	13	8		18	16	16	10	16	18	19	10		16	16	16
Viable Test	15	—	5	—	—	—	—	—	15	—	21	18	—	—	—	19
	16		1						17		20	12		—	—	18
Abiotic Background	—	—	—	17	—	—	—	—	5	—	—	—	—	—	—	16
				17					19							10
Abiotic Test	—	—	—	14	—	—	—	—	4	—	—	—	—	—	—	16
				14					14							14

Note: Headspace of viable microcosms exchanged with ambient air after 21, 51, and 92 days of incubation.

^aNo measurement made

Table 12. Thickness of Water and Sediment Layers in Selected Microcosms

Incubation Time (days) ^a :	Description	14 days		Ratio ^b	n	119 days			% Change	
		Water Depth (cm)	Sediment Depth (cm)			Water Depth (cm)	Sediment Depth (cm)	n	Water	Sediment
Schuylkill River – Viable	Aerobic	4.1 ± 0.1 ^c	1.5 ± 0.1	2.7	4	4.0 ± 0.0	1.5 ± 0.0	5	-2	0
Schuylkill River - Abiotic		4.0 ± 0.0	1.4 ± 0.1	2.9	4	4.0 ± 0.0	1.5 ± 0.0	2	0	7
Neshaminy Creek - Viable		3.5 ± 0.1	2.2 ± 0.2	1.6	4	3.8 ± 0.0	2.2 ± 0.2	5	+9	0
Neshaminy Creek- Abiotic		3.7 ± 0.0	2.0 ± 0.0	1.9	4	3.8 ± 0.1	2.0 ± 0.1	2	+3	0
Schuylkill River - Viable	Anaerobic	3.8 ± 0.2	1.8 ± 0.2	2.1	4	3.9 ± 0.1	1.7 ± 0.1	4	+3	-6
Schuylkill River - Abiotic		4.0 ± 0.0	1.5 ± 0.0	2.7	4	4.0 ± 0.0	1.6 ± 0.1	4	0	+7
Neshaminy Creek - Viable		3.6 ± 0.2	1.9 ± 0.1	1.9	4	4.0 ± 0.0	1.7 ± 0.1	4	11	-11
Neshaminy Creek - Abiotic		4.0 ± 0.0	1.5 ± 0.0	2.7	4	4.0 ± 0.0	1.7 ± 0.1	3	0	+13

^aIncubation time following the addition of HBCD

^bRatio of thickness of water/sediment layers

^c± 1 Standard deviation

Table 13. HBCD Concentrations in Aerobic Schuylkill River Microcosms

Nominal HBCD concentration: 34 ng/g

Day	Viable			Abiotic		
	HBCD (ng/g) ^a	% Decrease	Recovery Factor ^b (%)	HBCD (ng/g) ^a	% Decrease	Recovery Factor ^b (%)
0	31.9 ± 2.5 ^{c,d}	0	83	31.3 ± 0.5 ^c	0	83 ^e
1	11.2 ± 3.4 ^c	65	112	— ^f		—
7	31.2 ± 0.3 ^c	22	42	—		—
21	3.2 ± 1.2	90	98 ± 9	29.0 ± 2.7	7	92 ± 0
47	1.0 ± 0.8	97	56 ± 2	—		—
64	nd ^g	>98	59 ± 3	21.6 ± 3.3	31	58 ± 5
91	nd	>98	67 ± 1	—		—
119	3.3 ± 4.6	90	57 ± 2	20.7 ± 0.7	34	55 ± 5

^aBased on dry weight of sediment

^bPercent recovery factor (determined from sediment matrix spike sample) used to correct reported HBCD value.

^cValue includes HBCD contribution from the water phase:

- Day 0 Viable – 16%
- Day 0 Abiotic – 14%
- Day 1 Viable – 20%
- Day 7 Viable – 7%

^d± 1 Standard deviation

^ePercent recovery factor applied from viable, Day 0 matrix spikes prepared and analyzed on the same day.

^fMeasurement not made.

^gnd- Not detected; detection limit 0.5 ng/g

Table 14. HBCD Concentrations in Aerobic Neshaminy Creek Microcosms

Nominal HBCD concentration: 60 ng/g

Day	Viable			Abiotic		
	HBCD (ng/g) ^a	% Decrease	Recovery Factor ^b (%)	HBCD (ng/g) ^a	% Decrease	Recovery Factor ^b (%)
0	80.7 ± 5.8 ^{c,d}	0	55	58.3 ± 10.0	0	55 ^e
1	42.4 ± 9.5 ^c	47	60	— ^f	—	—
7	19.1 ± 9.3	76	55	—	—	—
21	5.7 ± 2.9	93	75 ± 10	22.0 ± 10.7	62	69 ± 10
64	3.0 ± 3.4	96	92 ± 3	31.1 ± 4.7	47	60 ± 13
91	0.6 ± 0.9	>99	60 ± 1	—	—	—
119	9.2 ± 4.1	89	77 ± 5	2.7 ± 3.8	95	72 ± 7

^aBased on dry weight of sediment

HBCD concentrations corrected for interference measured in background microcosms:

Viable – 19.5 ± 3.0 ng/g (Day 0, 7, and 119 samples)

Abiotic – 5.2 ng/g (Day 0 sample)

^bPercent recovery factor (determined from sediment matrix spike sample) used to correct reported HBCD value.

^c Value includes HBCD contribution from the water phase:

- Day 0 Viable – 22%
- Day 0 Abiotic – 18%
- Day 1 Viable – 4%

^d ± 1 Standard deviation

^ePercent recovery factor applied from viable, day 0 matrix spikes prepared and analyzed on the same day.

^fNo measurement made

Table 15. HBCD Concentrations in Anaerobic Schuylkill River Microcosms

Nominal concentration: 63 ng/g

Day	Viable			Abiotic		
	HBCD (ng/g) ^a	% Decrease	Recovery Factor ^b (%)	HBCD (ng/g) ^a	% Decrease	Recovery Factor ^b (%)
0	27.7 ± 6.7 ^{c,d}	0	56	27.2 ± 1.6	0	56 ^e
1	6.2 ± 1.4	78	125	— ^f	—	—
7	nd ^g	>98	33	—	—	—
14	nd	>98	38 ± 0	14.2 ± 0.3	48	34 ± 0
61	nd	>98	82 ± 8	nd	>98	65 ± 6
91	nd	>98	70 ± 3	—	—	—
119	nd	>98	56	nd	>98	56 ± 4

^aBased on dry weight of sediment

^bPercent recovery factor (determined from sediment matrix spike sample) used to correct reported HBCD value.

^c Value includes HBCD contribution from the water phase:

- Day 0 Viable – 20%

^d ± 1 Standard deviation

^ePercent recovery factor applied from viable, Day 0 matrix spikes prepared and analyzed on the same day.

^fMeasurement not made

^gnd- Not detected; detection limit 0.5 ng/g

Table 16. HBCD Concentrations in Anaerobic Neshaminy Creek Microcosms

Nominal HBCD concentration: 89 ng/g

Day	Viable			Abiotic		
	HBCD (ng/g) ^a	% Decrease	Recovery Factor ^b (%)	HBCD (ng/g) ^a	% Decrease	Recovery Factor ^b (%)
0	39.1 ± 5.4 ^{c,d}	0	108	37.5 ± 6.6 ^c	0	108 ^e
1	55.9 ± 18.8 ^c	0	56	– ^f	–	–
7	nd ^g	>98		–	–	–
14	nd	>98	57 ± 1	14.2 ± 6.3	62	67 ± 7
62	nd	>98	76 ± 1	nd	>98	67 ± 8
91	nd	>98	69 ± 1	–	–	–
119	nd	>98	63	1.4 ± 2.0	96	70

^aBased on dry weight of sediment

HBCD concentrations corrected for interference measured in background microcosms:

Viable – 24.7 ± 1.3 ng/g (Day 0, 1, and 119 samples)

Abiotic – 4.9 ng/g (Day 0 sample)

^bPercent recovery factor (determined from sediment matrix spike sample) used to correct reported HBCD value.

^c Value includes HBCD contribution from the water phase:

- Day 0 Viable – 6%
- Day 0 Abiotic – 20%
- Day 1 Viable – 2%

^d ± 1 Standard deviation

^ePercent recovery factor applied from viable, Day 0 matrix spikes prepared and analyzed on the same day.

^fMeasurement not made

^gnd- Not detected; detection limit 0.5 ng/g

Table 17. Pseudo-First Order Rate Constants and Half-lives for Transformation of HBCD in Sediment Microcosms

Microcosm Description	<u>Biotransformation</u>		<u>Abiotic (Control)</u>	
	k (days ⁻¹)	t _{1/2} (days)	k (days ⁻¹)	t _{1/2} (days)
Aerobic Schuylkill River	0.066	11	0.0037	190
Aerobic Neshaminy Creek	0.022	32	0.023	30
Anaerobic Schuylkill River	0.45	1.5	0.067	10
Anaerobic Neshaminy Creek	0.61	1.1	0.070	9.9

Note: 1) Rate constants for Neshaminy Creek microcosms were determined using HBCD concentrations corrected for the background interference present in microcosms.
2) Biotransformation rate constant determined by subtracting abiotic rate constant from viable rate constant.

Viable rate constants used (see Appendices G and H) : Aerobic Schuylkill River : 0.070 days⁻¹

Aerobic Neshaminy Creek : 0.045 days⁻¹

Anaerobic Schuylkill River : 0.52 days⁻¹

Anaerobic Neshaminy Creek : 0.68 days⁻¹

Half-life (t_{1/2}) calculated from the relationship $t_{1/2} = \ln(2)/k$

Figure 1. Representative LC-MS Spectra for HBCD Analysis using APPI. A) 640.7 m/z; B) 79 m/z

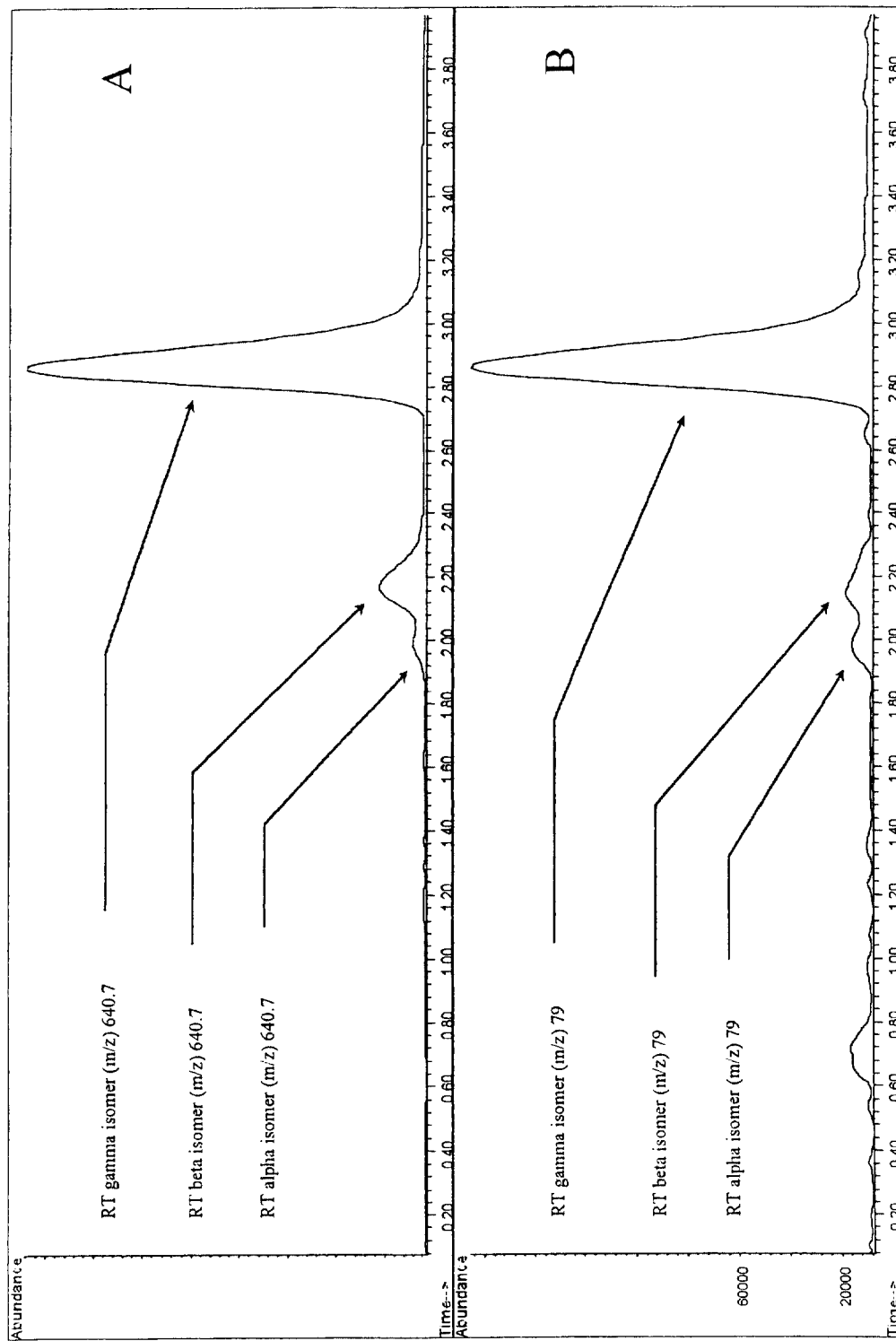


Figure 2. HBCD Concentrations in Aerobic Schuylkill River Microcosms

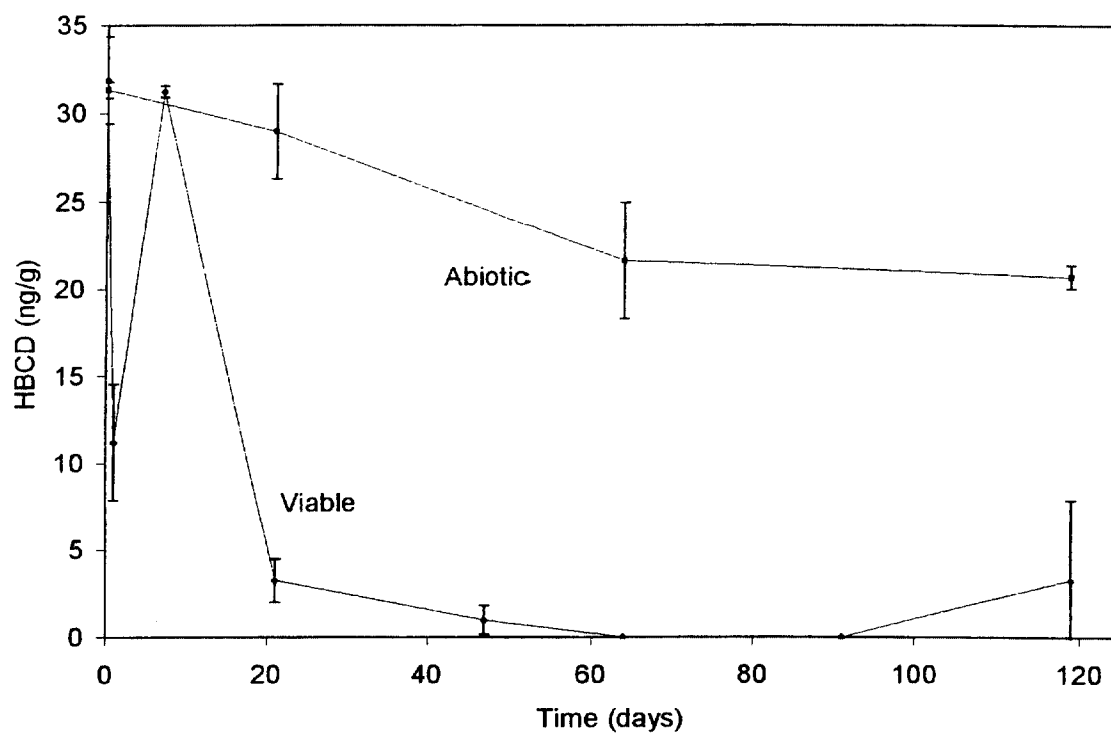


Figure 3. LC-MS Chromatogram for Day 0 Analysis of HBCD in Aerobic Schuylkill River Sediment Microcosm. A) 640.7 m/z; B) 79 m/z

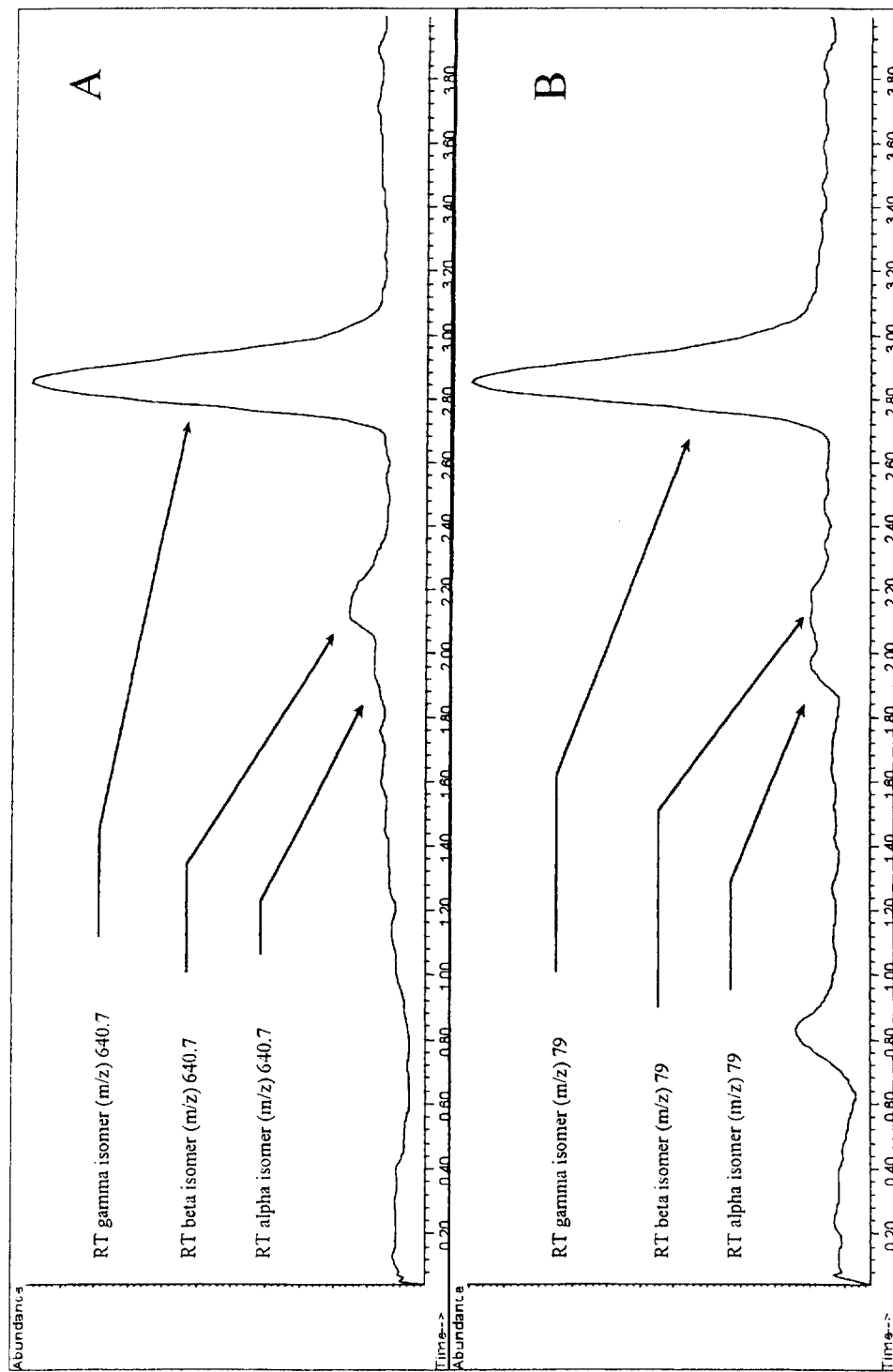


Figure 4. LC-MS Chromatogram for Day 21 Analysis of HBCD in Aerobic Schuylkill River Sediment Microcosm. A) 640.7 m/z; B) 79 m/z

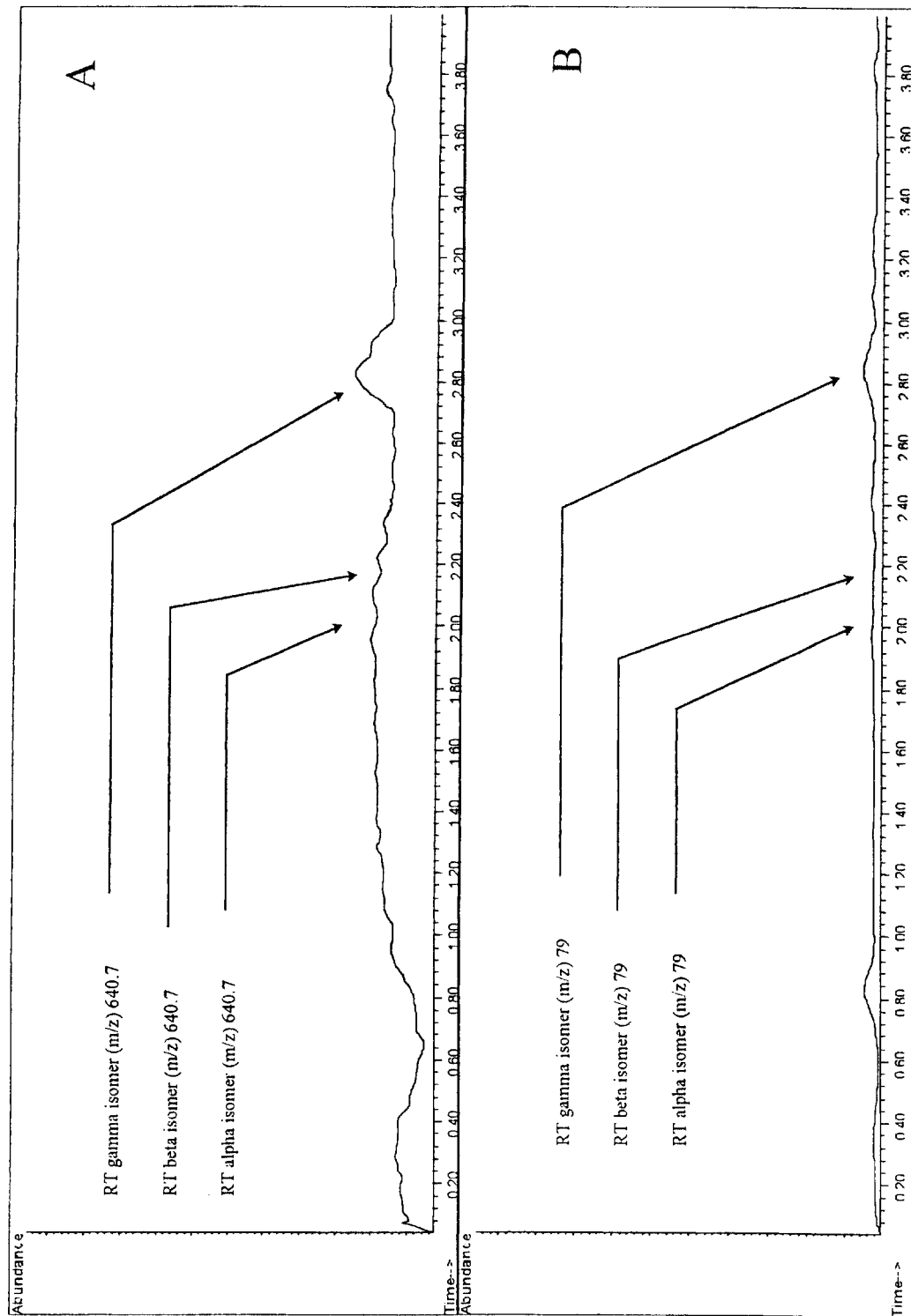


Figure 5. LC-MS Chromatogram for Day 64 Analysis of HBCD in Aerobic Schuylkill River Sediment Microcosm. **A)** 640.7 m/z; **B)** 79 m/z

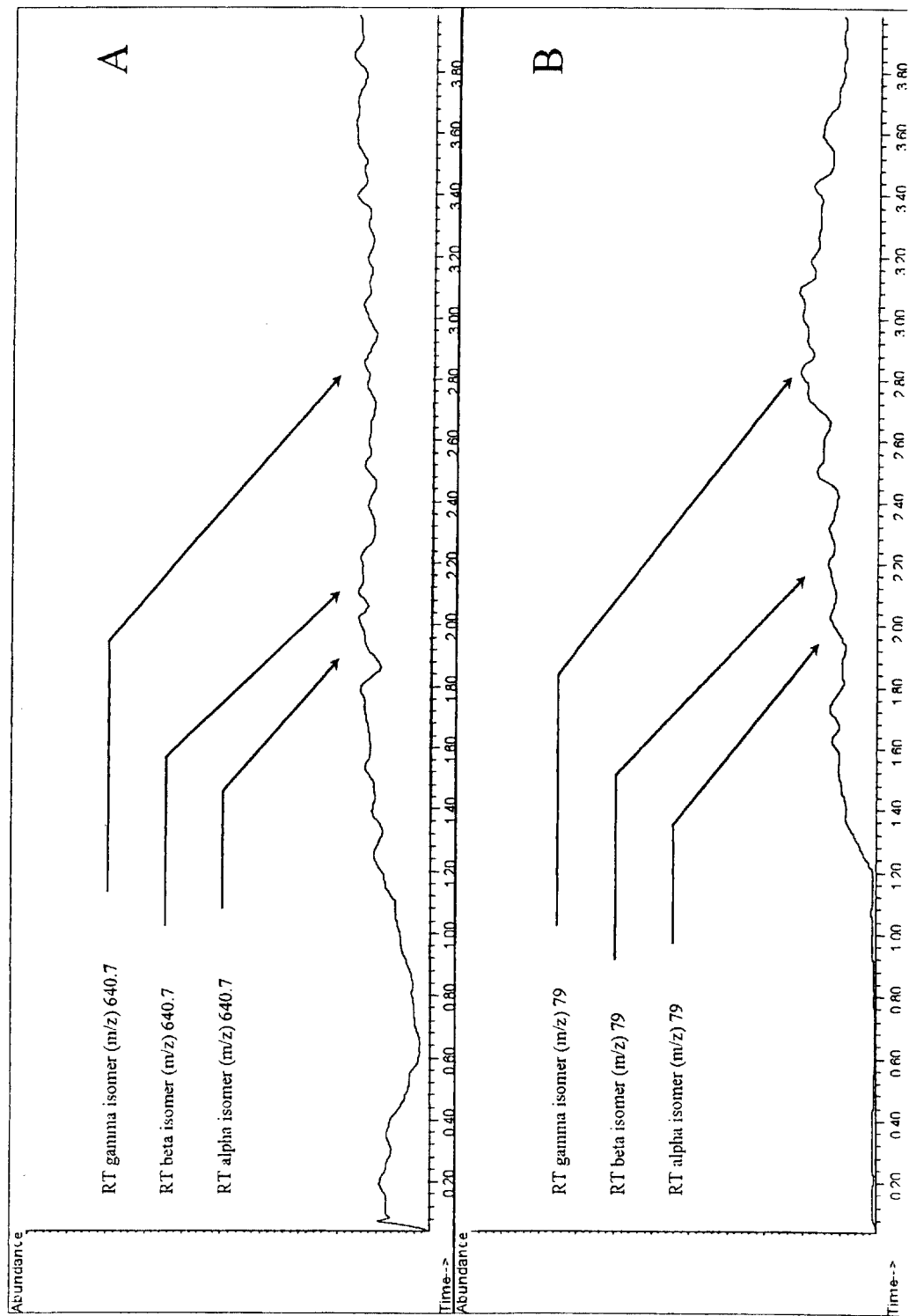


Figure 6. LC-MS Chromatogram for Day 64 Analysis of HBCD in Abiotic Aerobic Schuylkill River Sediment Microcosm. **A)** 640.7 m/z; **B)** 79 m/z

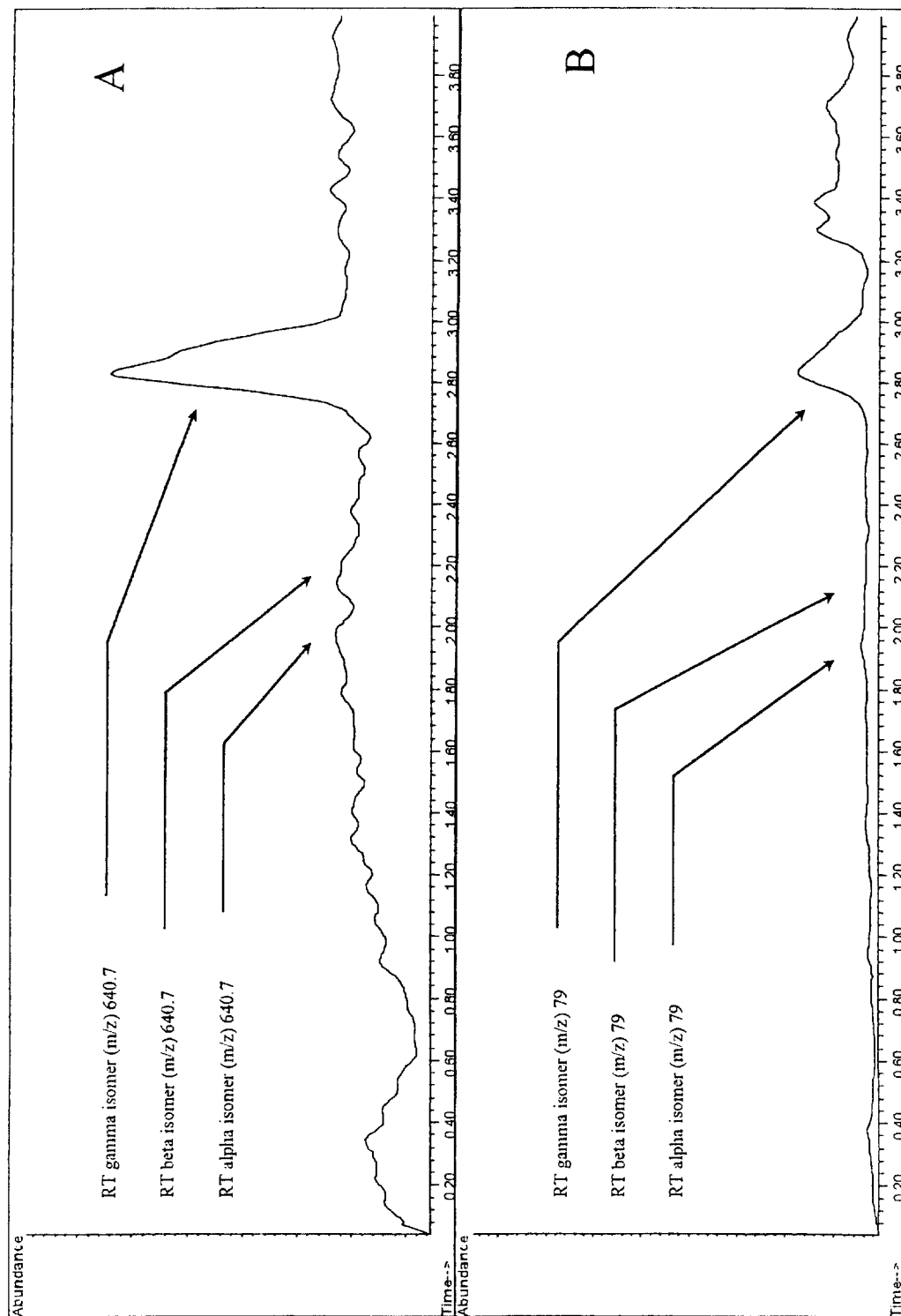


Figure 7. HBCD Concentrations in Aerobic Neshaminy Creek Microcosms

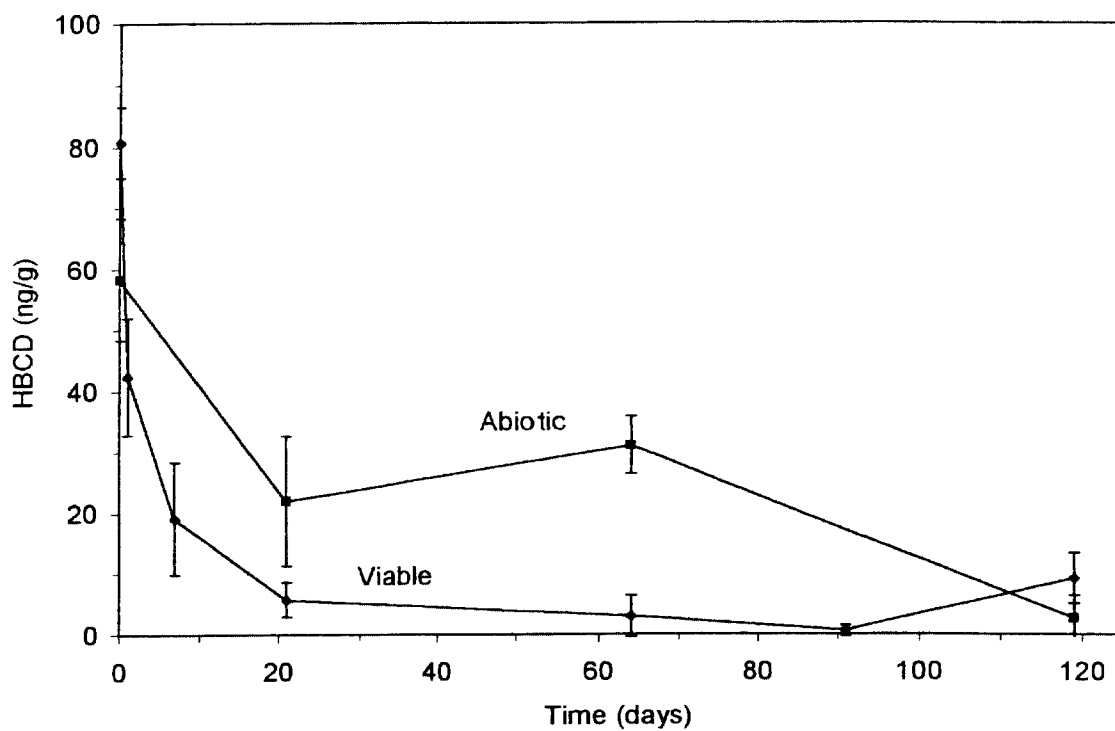


Figure 8. LC-MS Chromatogram for Day 0 Analysis of HBCD in Aerobic Neshaminy Creek Sediment Microcosm. A) 640.7 m/z; B) 79 m/z

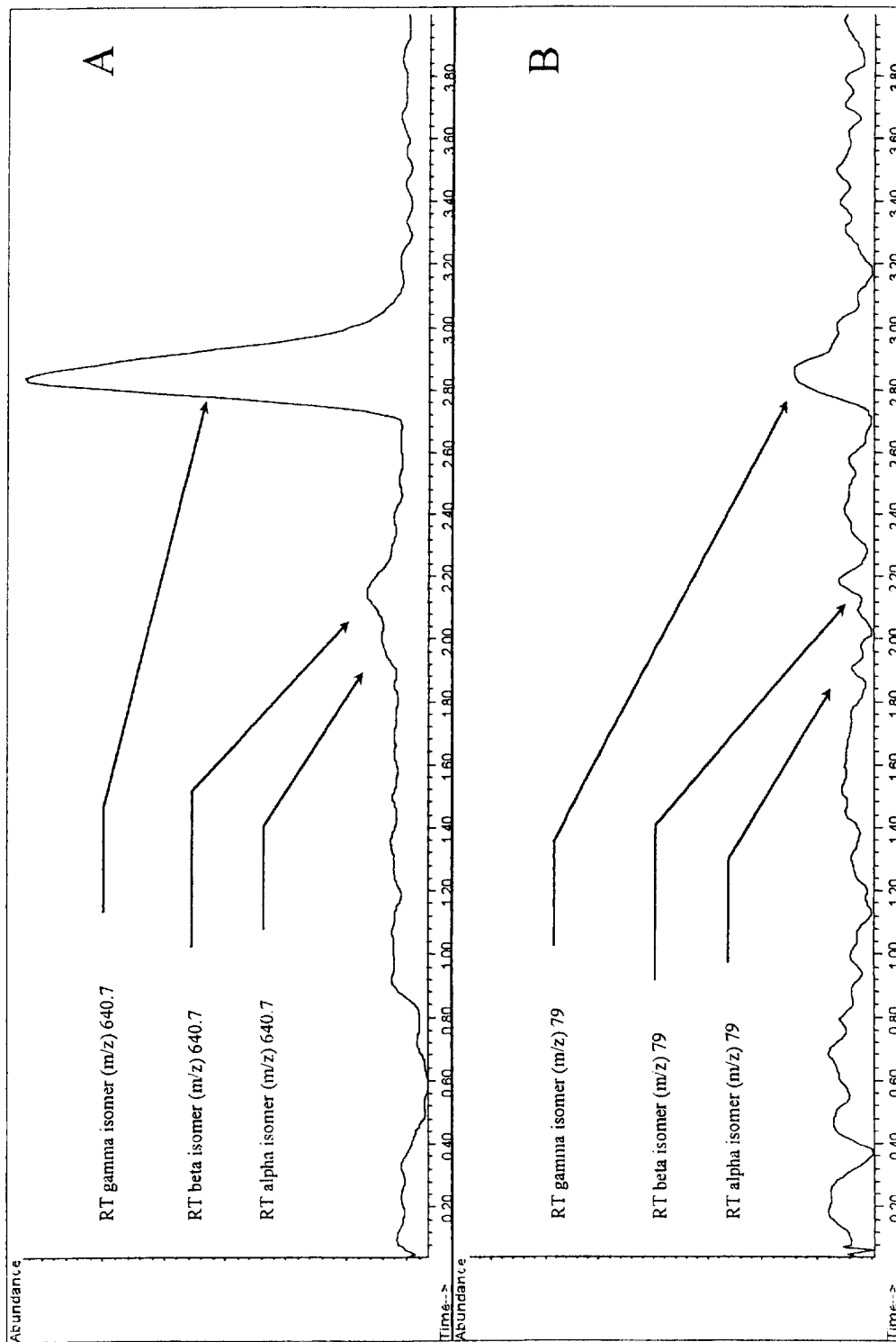


Figure 9. LC-MS Chromatogram for Day 91 Analysis of HBCD in Aerobic Neshaminy Creek Sediment Microcosm. A) 640.7 m/z; B) 79 m/z

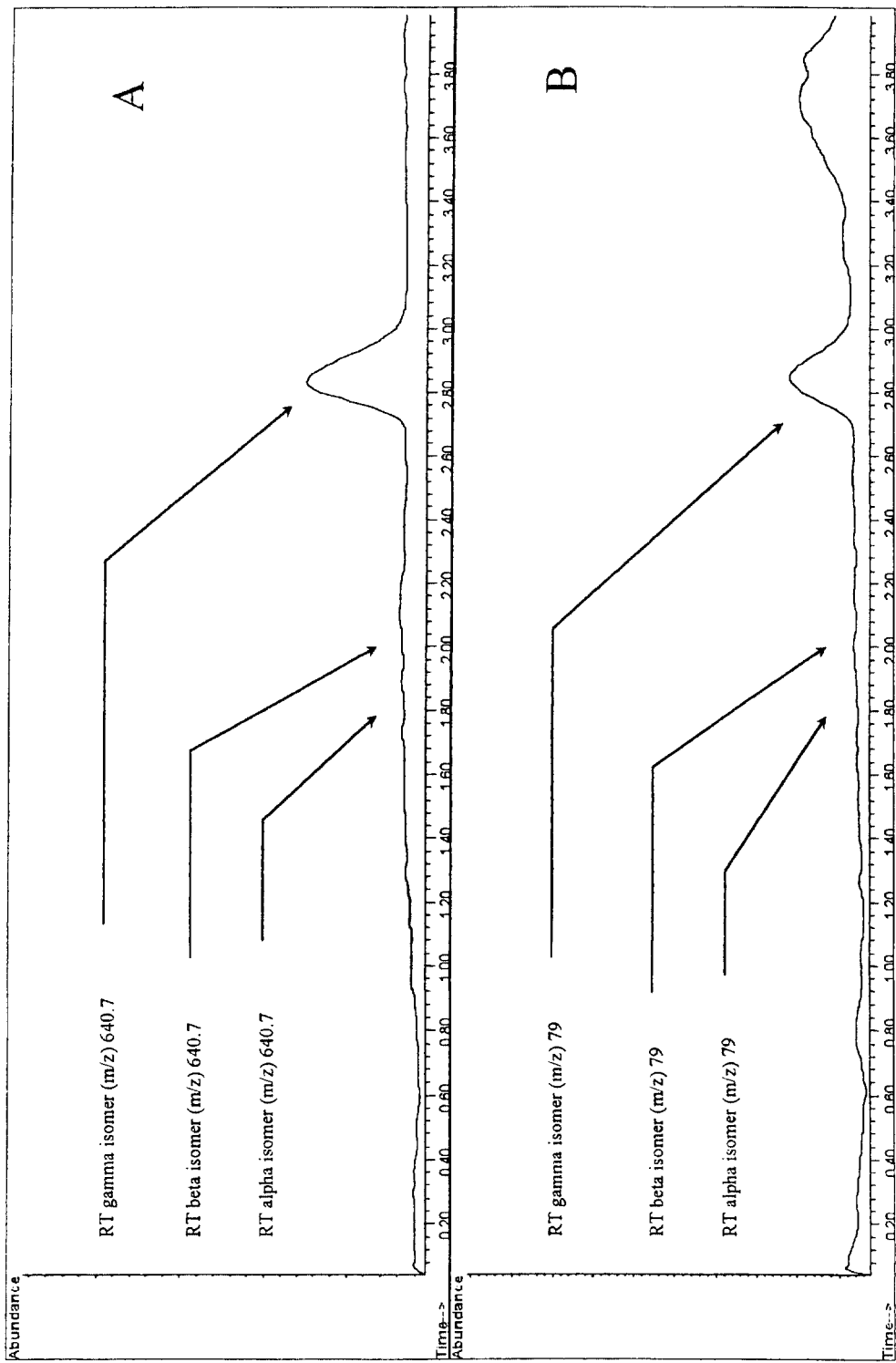


Figure 10. LC-MS Chromatogram for Day 0 Analysis of Background Aerobic Neshaminy Creek Sediment Microcosm. **A)** 640.7 m/z; **B)** 79 m/z

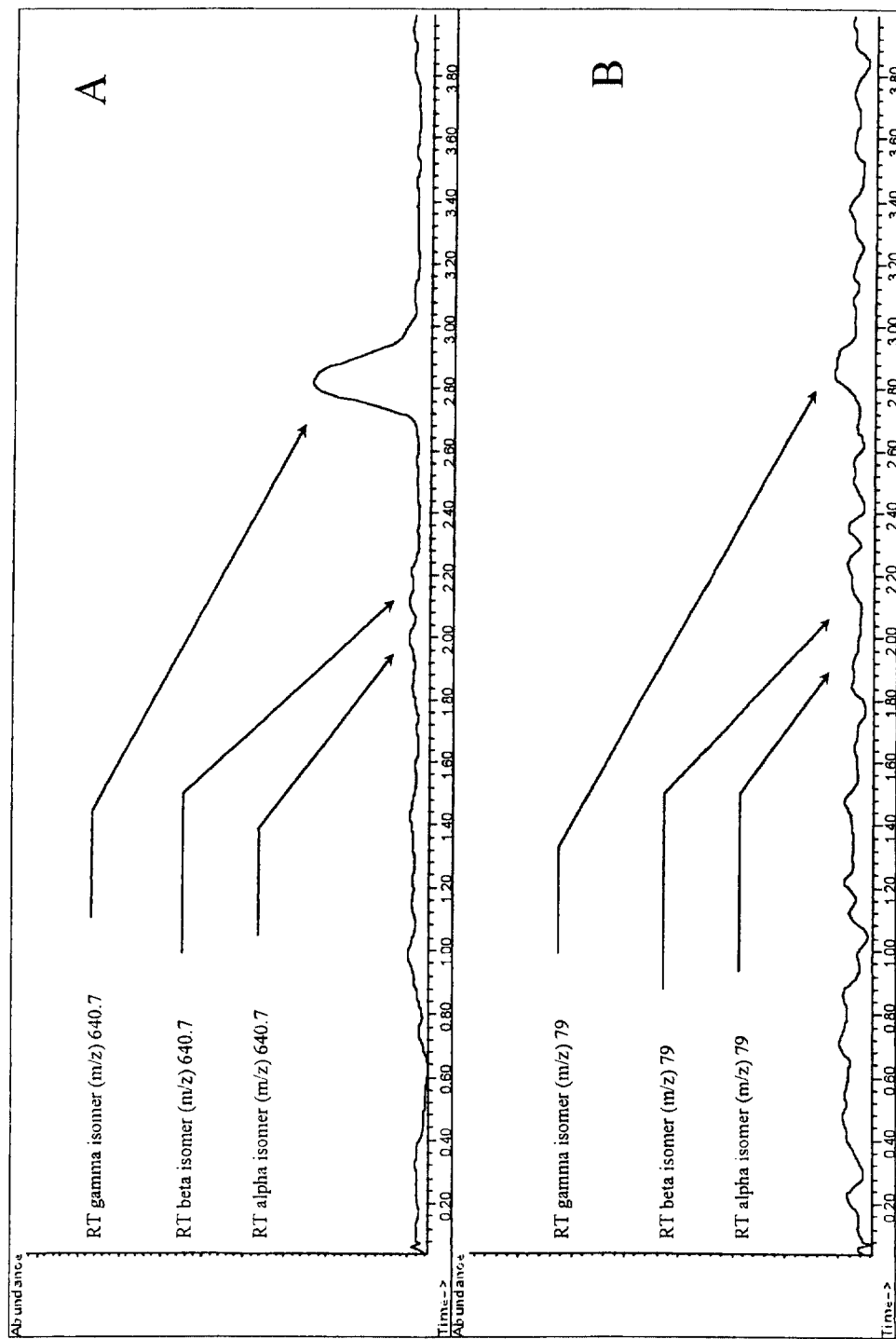


Figure 11. HBCD Concentrations in Anaerobic Schuylkill River Microcosms

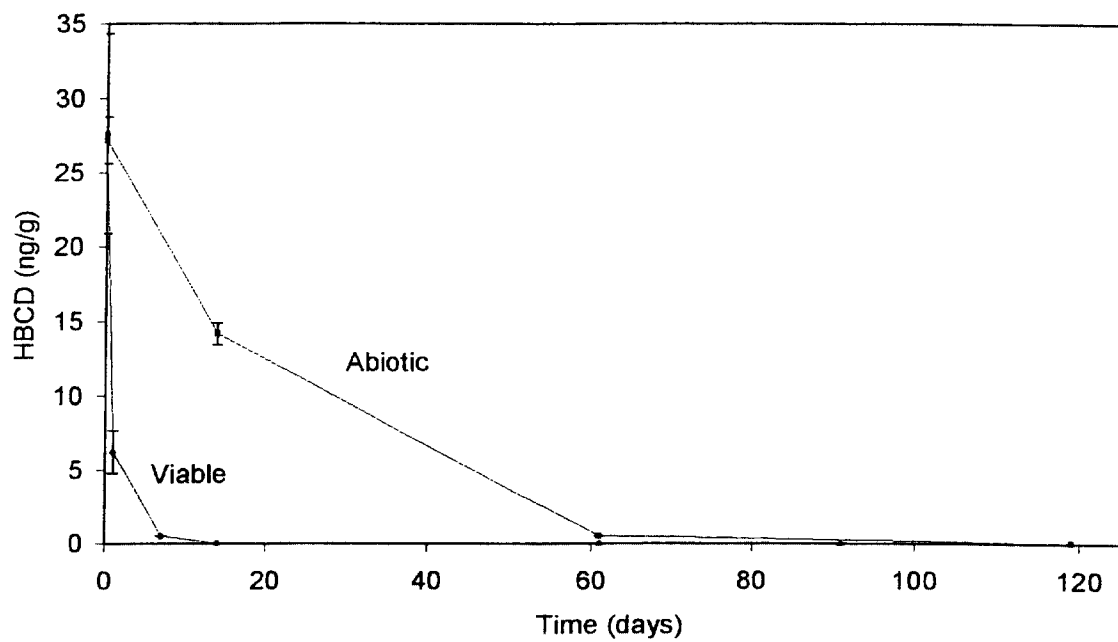


Figure 12. LC-MS Chromatogram for Day 0 Analysis of HBCD in Anaerobic Schuylkill River Sediment Microcosm. A) 640.7 m/z; B) 79 m/z

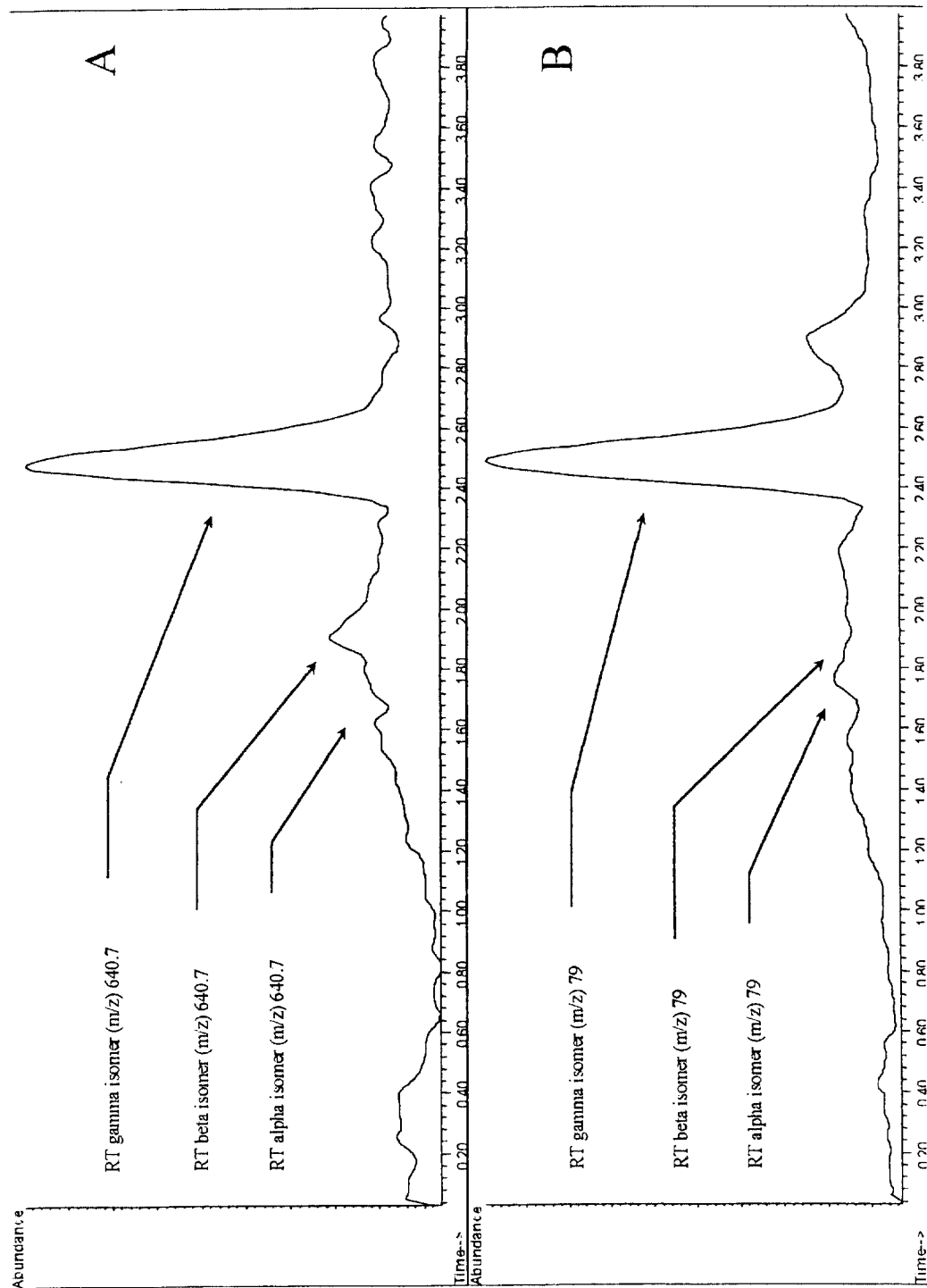


Figure 13. LC-MS Chromatogram for Day 14 Analysis of HBCD in Anaerobic Schuylkill River Sediment Microcosm. A) 640.7 m/z; B) 79 m/z

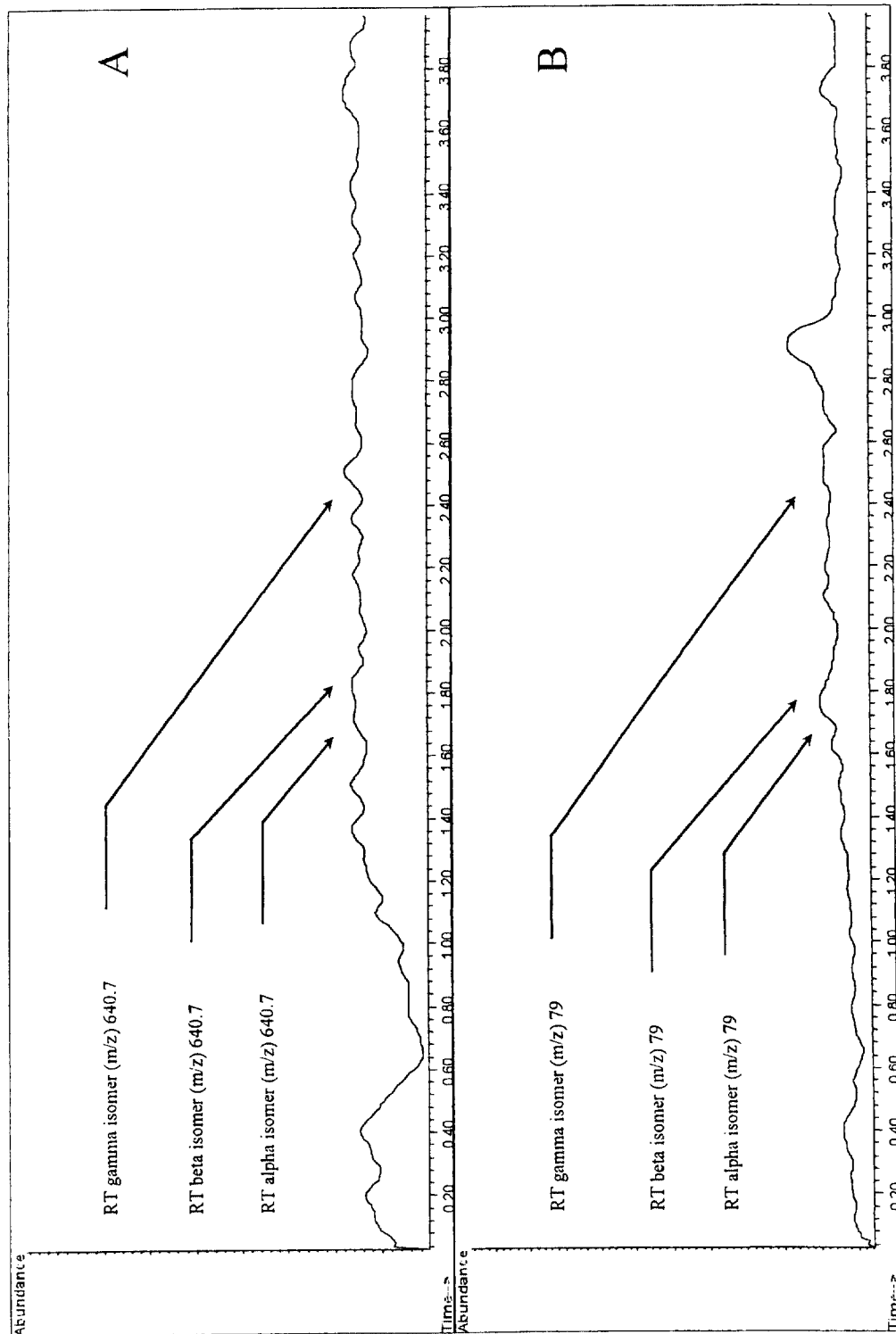


Figure 14. HBCD Concentrations in Anaerobic Neshaminy Creek Microcosms

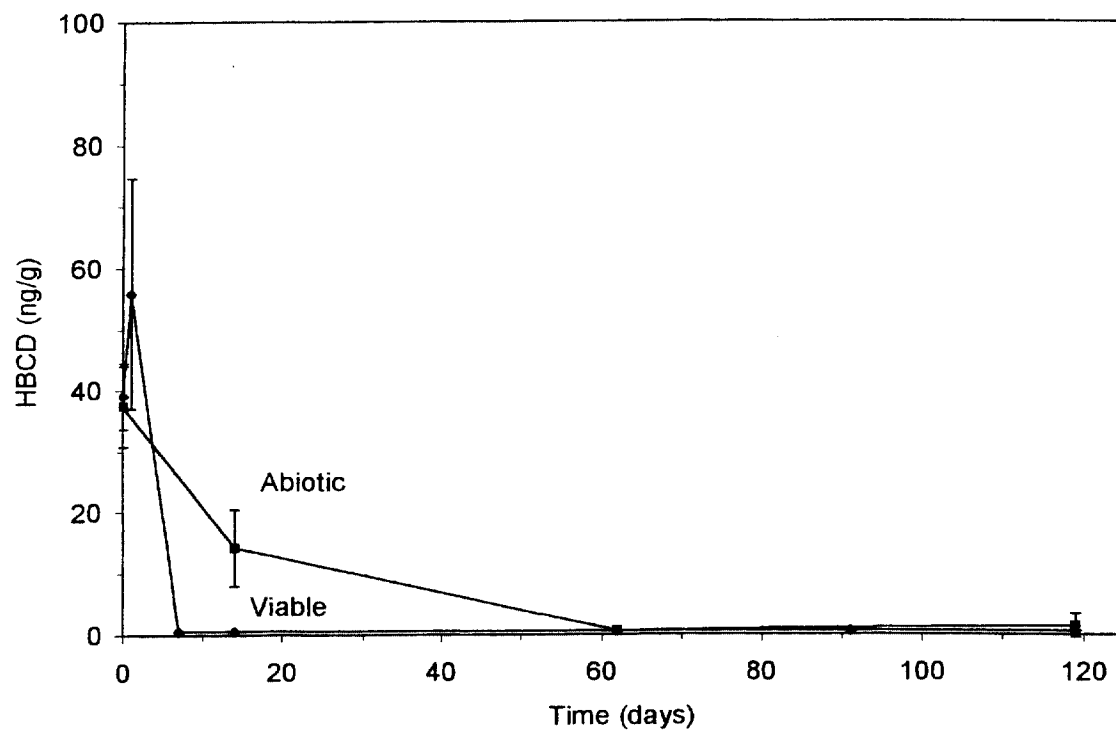


Figure 15. C-MS Chromatogram for Day 0 Analysis of HBCD in Anaerobic Neshaminy Creek Sediment Microcosm. **A)** 640.7 m/z; **B)** 79 m/z

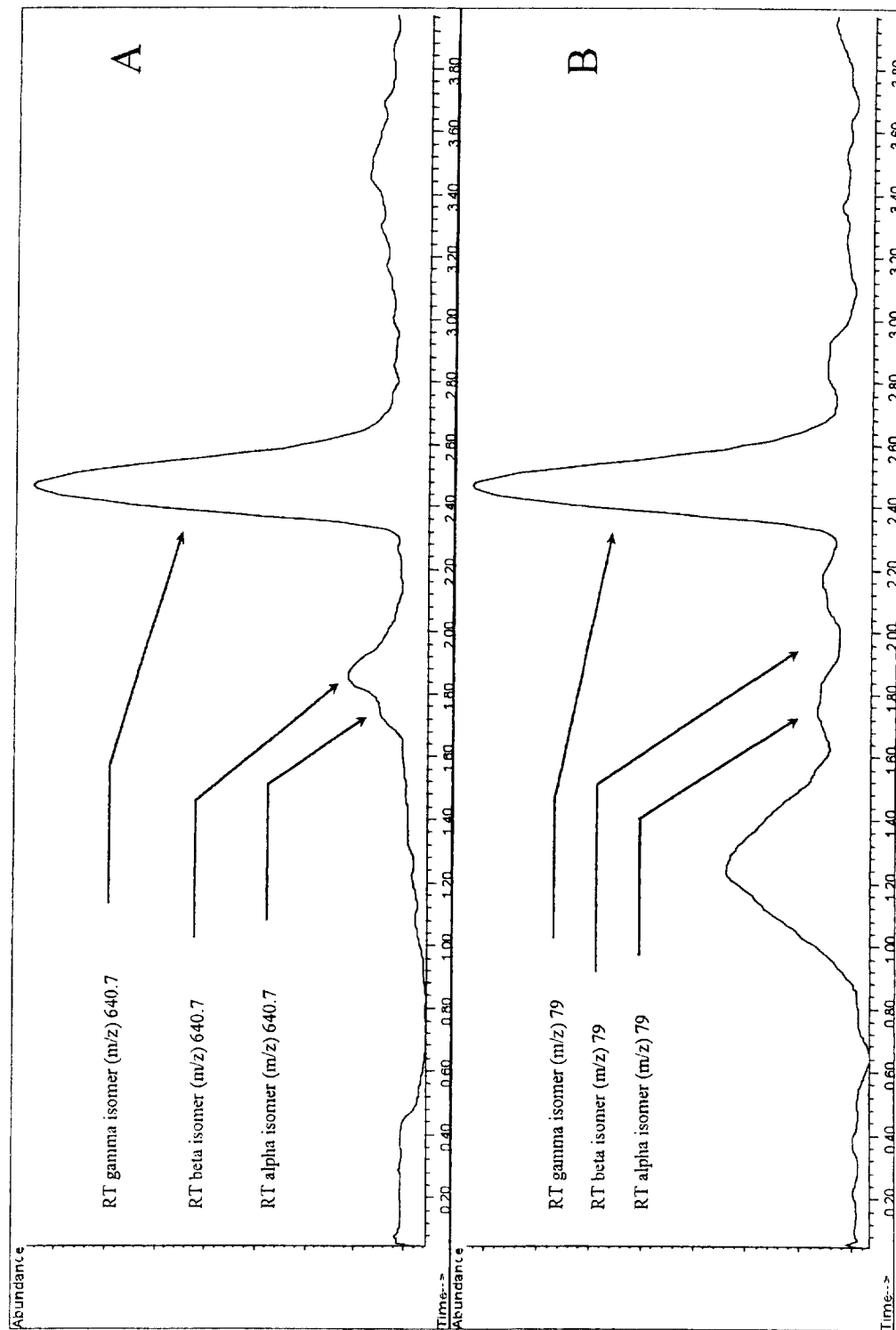


Figure 16. LC-MS Chromatogram for Day 119 Analysis of HBCD in Anaerobic Neshaminy Creek Sediment Microcosm. A) 640.7 m/z; B) 79 m/z

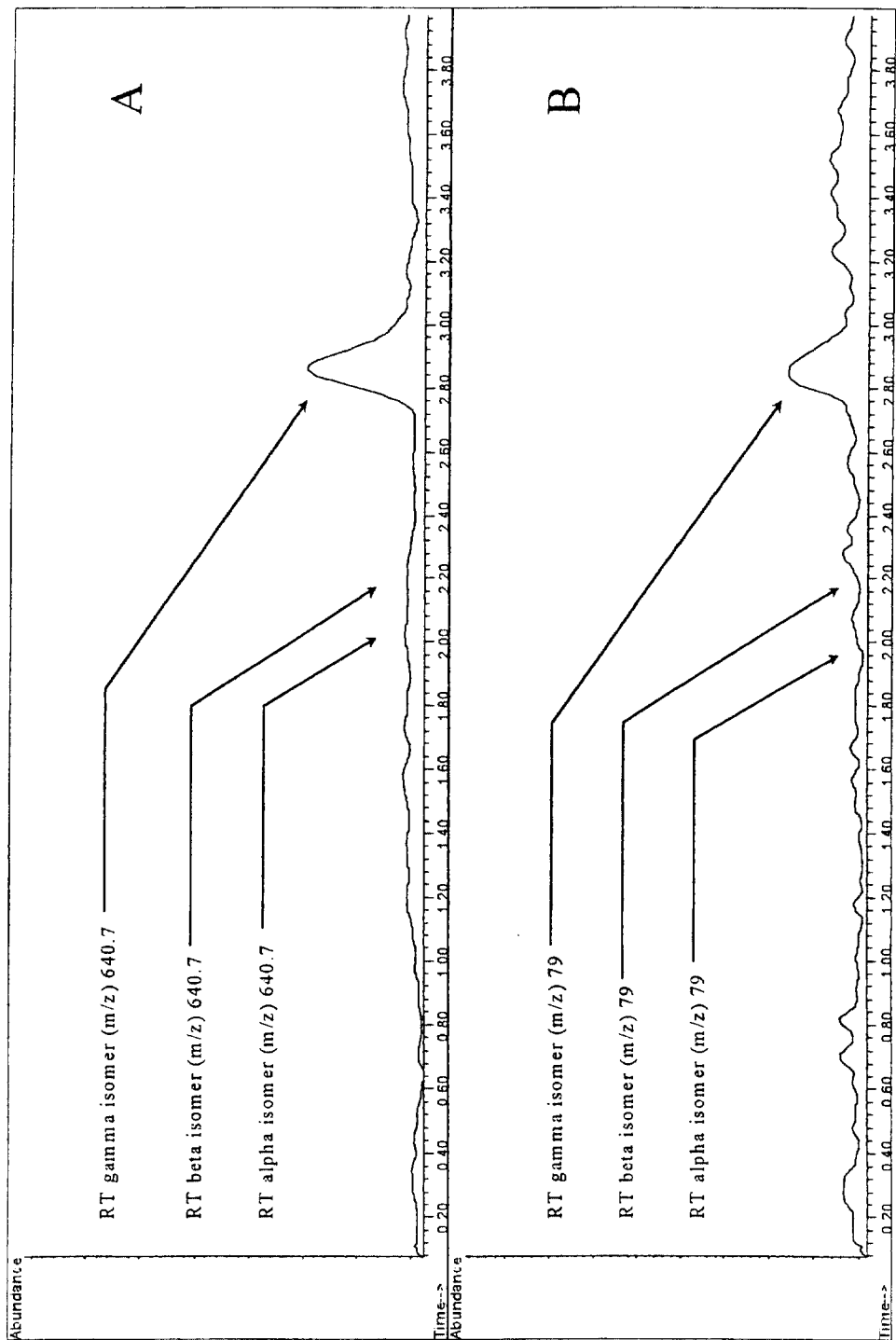
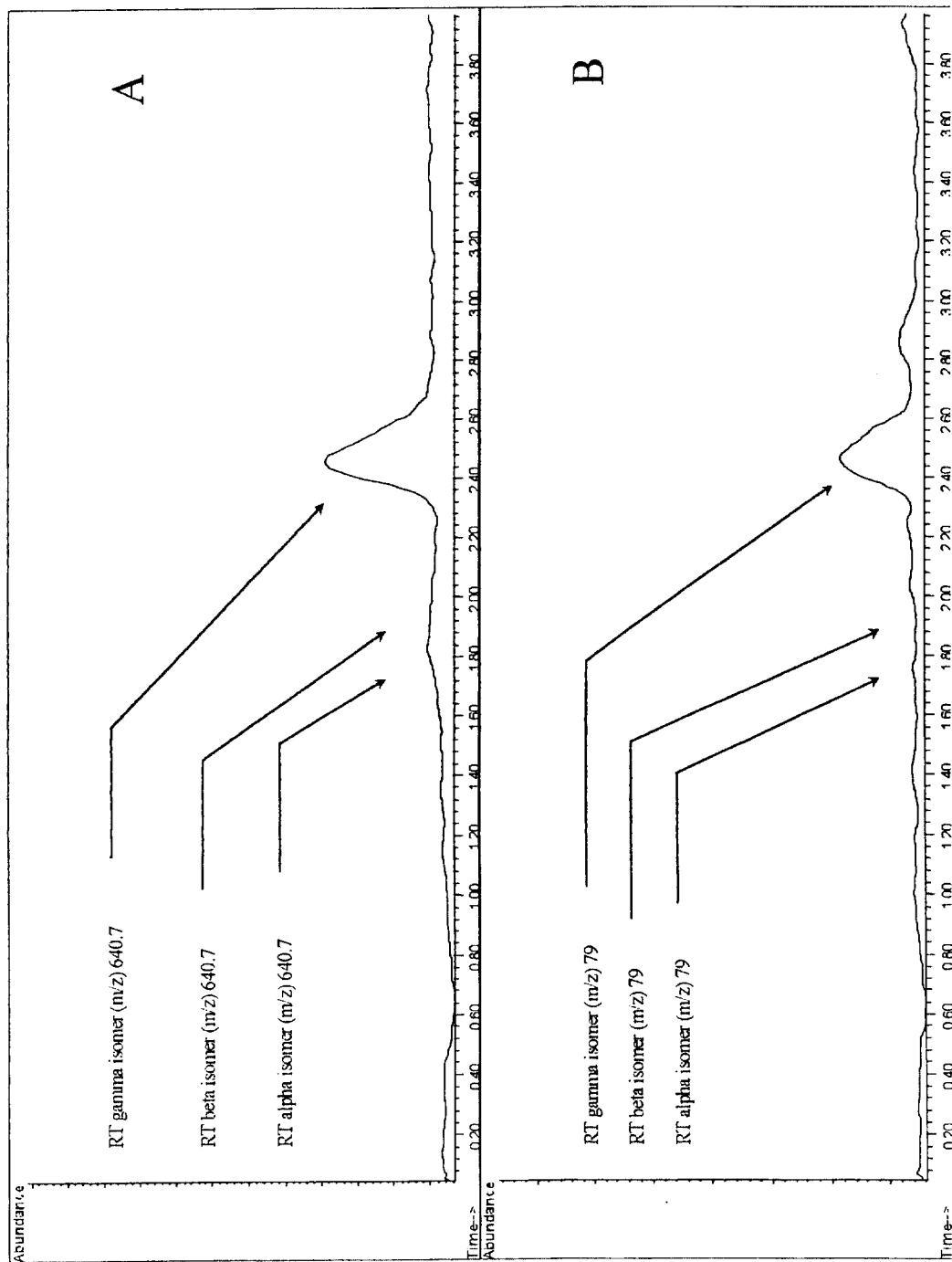


Figure 17. LC-MS Chromatogram for Day 0 Analysis of Background Anaerobic Neshaminy Creek Sediment Microcosm. **A)** 640.7 m/z; **B)** 79 m/z



Appendix A. Preparation of Sediment Microcosm for LC-MS analysis of HBCD

- 1) Centrifuge microcosm at 1000 rpm for 10 minutes.
- 2) Decant water layer into 125-mL serum bottle pre-rinsed with hexanes.
- 3) Dry sediment samples with anhydrous sodium sulfate – 75 g for Schuylkill River sediments; 100 g for Neshaminy Creek sediments.
- 4) Add 100- mL hexanes to sediment sample and 30-mL hexanes to water sample and mix 1 day.
- 5) Centrifuge soil and water samples at 1000 rpm 10 minutes. (Note: Additional centrifugation at higher speeds (increments of 500 rpm up to 2000 rpm for 15 minutes) is needed for some water samples to reduce emulsions that are formed during extraction step)

Sediment Preparation:

- Hexanes extract of the sediment sample is decanted into a 40 mL vial.
- About 11 mL of the extract are transferred into a 20-mL vial containing 5 mL of Milli-Q[®] water. The remainder of the extract is saved as a retainer sample.
- The vial is capped and shaken vigorously. This wash step removes residual salts from the hexanes extract (Note: Failure to wash the extract sample results in the accumulation of salts in the LC-MS interface, resulting in a rapid decrease in instrument sensitivity). The washed extract is allowed to stand for several minutes to allow for complete separation of the organic and aqueous phases.
- Using a 10 mL disposable glass pipette, 9.5 mL of the washed extract is transferred into a 20 mL vial.
- The washed extract is evaporated to dryness in 50 to 60 minutes at medium heat in a speed vac.
- 1 to 2 mL of acidified diluent (Acetonitrile/Water (85/15) + 0.1% HCl) is added to the vial to dissolve the residue. The vial is capped and placed on a vortex mixer for approximately 1 minute. The reconstituted sample is transferred to a 2-mL vial, capped, and analyzed by LC-MS.

Water Sample Preparation:

- Hexanes extract of the water sample is decanted into a 20 mL vial.
- A 9.5-mL aliquot of the extract is transferred into a 20-mL vial and the volume is recorded. (Note: the extract is not washed since the water layer sample was not dried with sodium sulfate).
- The extracts are evaporated to dryness in 50 to 60 minutes at medium heat in a speed vac.
- 1 to 2-mL of acidified diluent (acetonitrile/water (85/15) + 0.1% HCl) is added to the vial to dissolve the residue. The vial is capped and placed on a vortex mixer for approximately 1 minute. The reconstituted sample is transferred to a 2-mL vial, capped and is analyzed by LC-MS.

Appendix B. HBCD LC/MS Conditions:

Agilent 1100 Series LC/MS

- 1100-SL Mass Selective Detector (MSD) - G1946D

- Group 1 – Select Ion Monitoring (SIM)

On at 0.0 minutes:

<u>SIM Ion</u>	<u>Fragmentor</u>	<u>Gain (EMV)</u>	<u>SIM Resolution</u>	<u>Dwell</u>
79	160	30.0	Low	289
640.7	160	30.0	Low	289

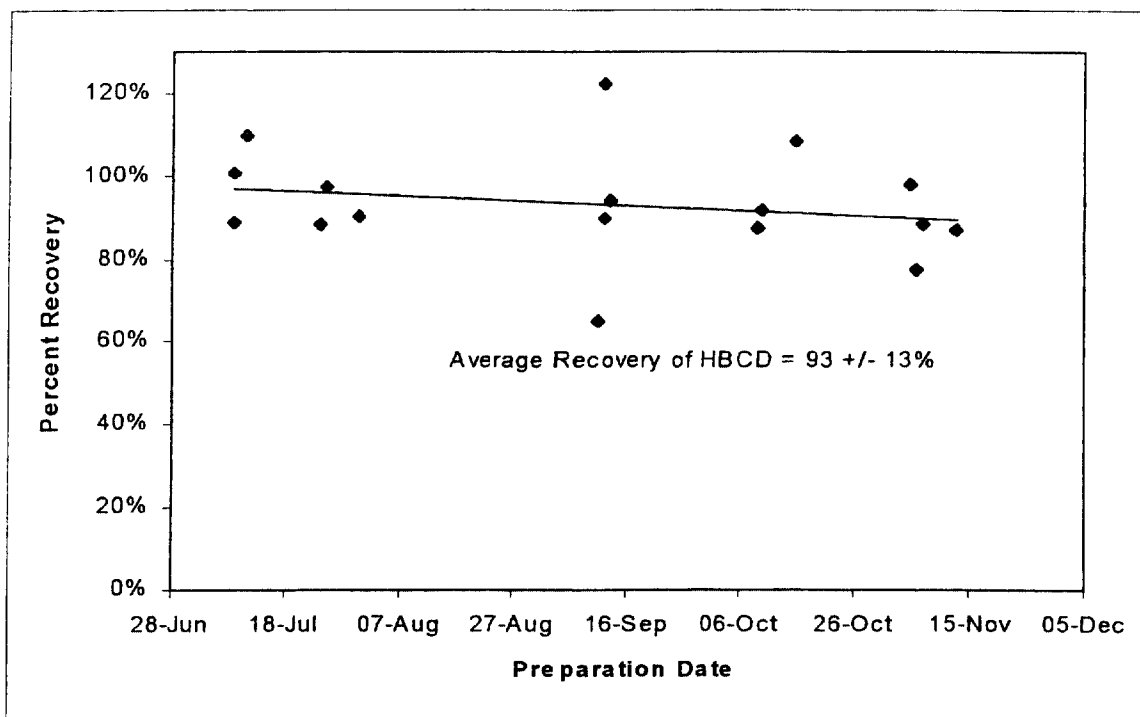
- Atmospheric Pressure Photo Ionization (APPI) Source: G1947-60101
 - Gas Temp: 250 °C.
 - Vaporizer Temp: 240 °C.
 - Dry Gas: 7.0 L / minute.
 - Nebulizer Pressure: 45 psig.
 - VCap (negative): 5000 V
- Bin Pump – G1312A.
 - Column Flow: 0.75 mL/min - Isocratic.
 - Stop Time: 4 minutes.
 - Solvent A-2: 15% Water + 0.1% Formic Acid.
 - Solvent B-2: 85% Acetonitrile + 0.1% Formic Acid.
- Auto sampler – G1313A.
 - Injection Volume: 100 uL.
- Column Oven - G1316A
 - Temperature Setting: Ambient.
- Variable Wavelength Detector (VWD) – G1314A.
 - Wavelength: 254 nm.
 - Peak width: > 0.1 minute.

Hitachi Model L-6200A LC (doping pump) – Pump Flow: 0.1 mL/minute.

Column: ODS-AQ 5u ; 2 x 150 mm

Data Acquisition: Agilent LC/MSD ChemStation – Rev A.08.04 [1008]

Appendix C. Recovery of HBCD in Method Spike Samples Over Time.



Appendix D. Gas Chromatography-Mass Spectrometry Instrument Conditions

Instrumentation

Agilent 6890 Gas Chromatograph (GC) -
Column: Agilent DB-624; 0.32 mm x 30 m; 1.8 μ m film
CTC Analytics – Leap GC Pal Auto sampler
Agilent 5973 Mass Selective Detector (MSD)

GC Conditions:

Injection Port

Injection volume (1 μ L)
Splitless mode
Injection Port Temperature – 250 °C
Pressure: 10 psi (helium carrier gas)
Purge Flow: 30 mL/min
Purge Time: 1 minute
Total Flow: 36.4 mL/min

Temperature Program

40 °C hold for 1 minute
Ramp @ 15 °C/minute to 200 °C
Hold 200 °C for 4 minutes

MSD conditions:

Solvent delay: 1 minute
EM offset: 200
Group 1 ions – low resolution (m/z): 79 and 81; dwell = 50
MS Quad temp: 150 °C
MS Source temp: 230 °C

Appendix E. Ion Chromatograph Conditions for Inorganic Bromide Analysis

Instrument: Dionex Model DX-120 Ion Chromatograph

Column: Dionex AS9-HC-4 mm (10-32)

Auto Sampler: Alcott Model 728R

Injection volume: 20 μ L

Mobile phase: 11 mM Na_2CO_3

Flowrate: 1.25 mL/minute

Pressure: 2476 psi

Total conductivity: 23.47 μ S

Offset: 0.076

Eluent: 11 mM Na_2CO_3

Perkin-Elmer TurboChrom[®] Data Acquisition System

Appendix F. Gas Chromatography-Flame Ionization Detection Instrument Conditions for Methane Analysis

Gas Chromatograph: Agilent model 6890

Column: PoraPlot Q-HT 0.53 mm x 25 m

Injection Port: 200 °C

Split mode, 50 cc/min split vent

7.5 psi head pressure

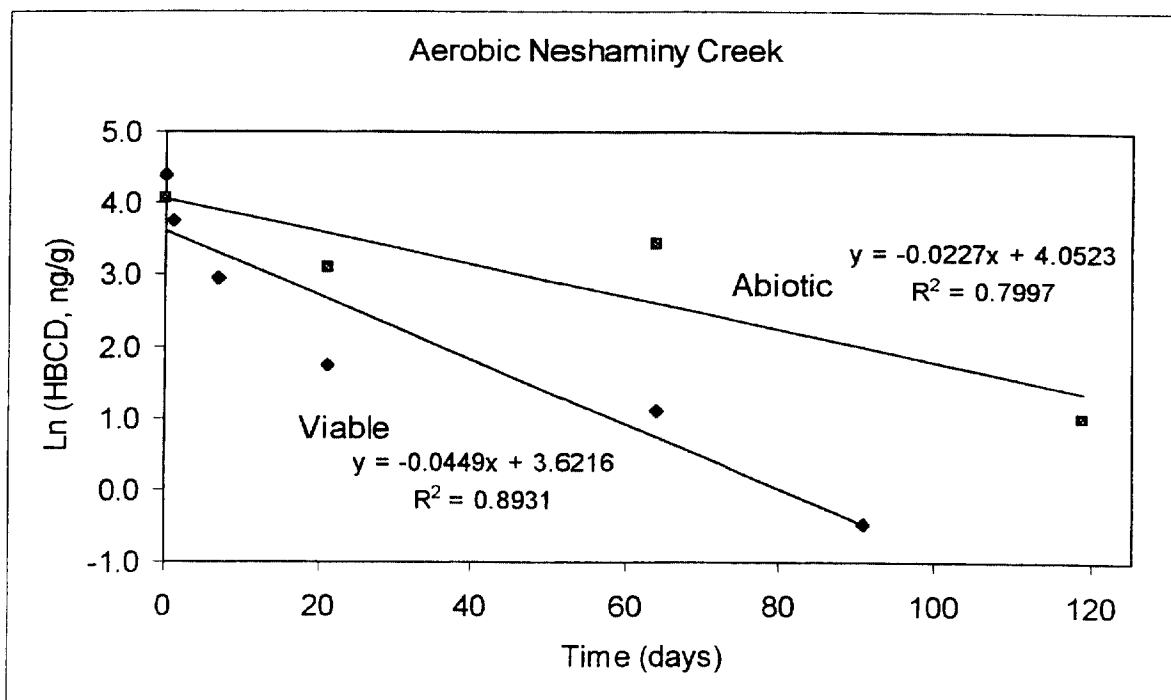
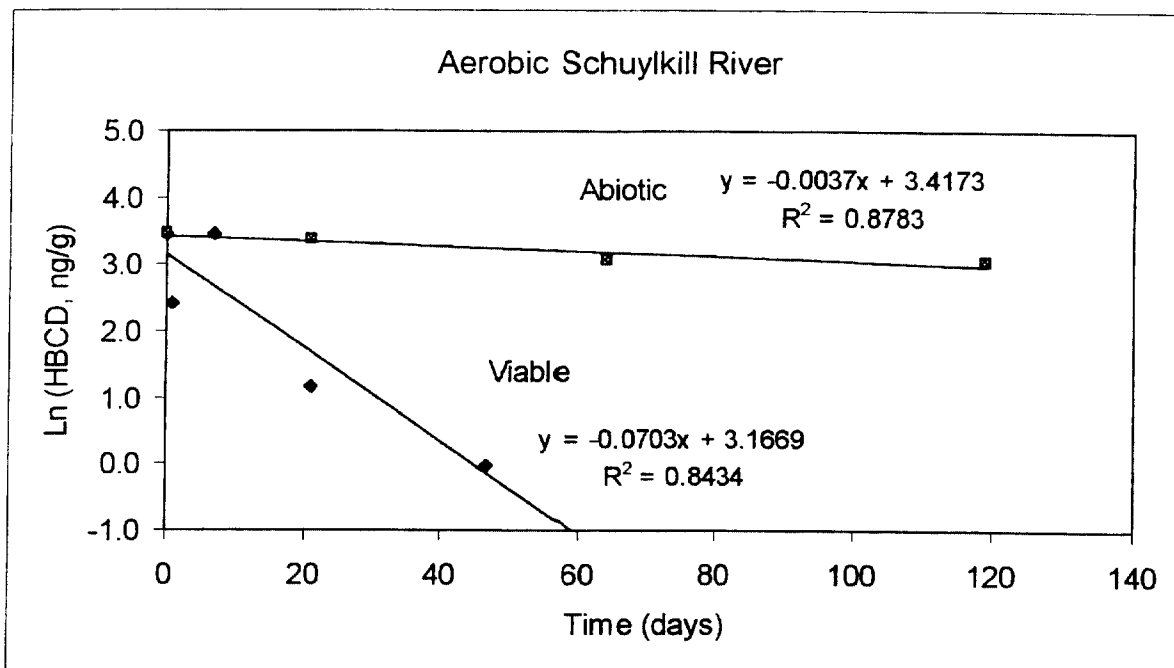
Carrier gas: Helium

Column: 45 °C isothermal

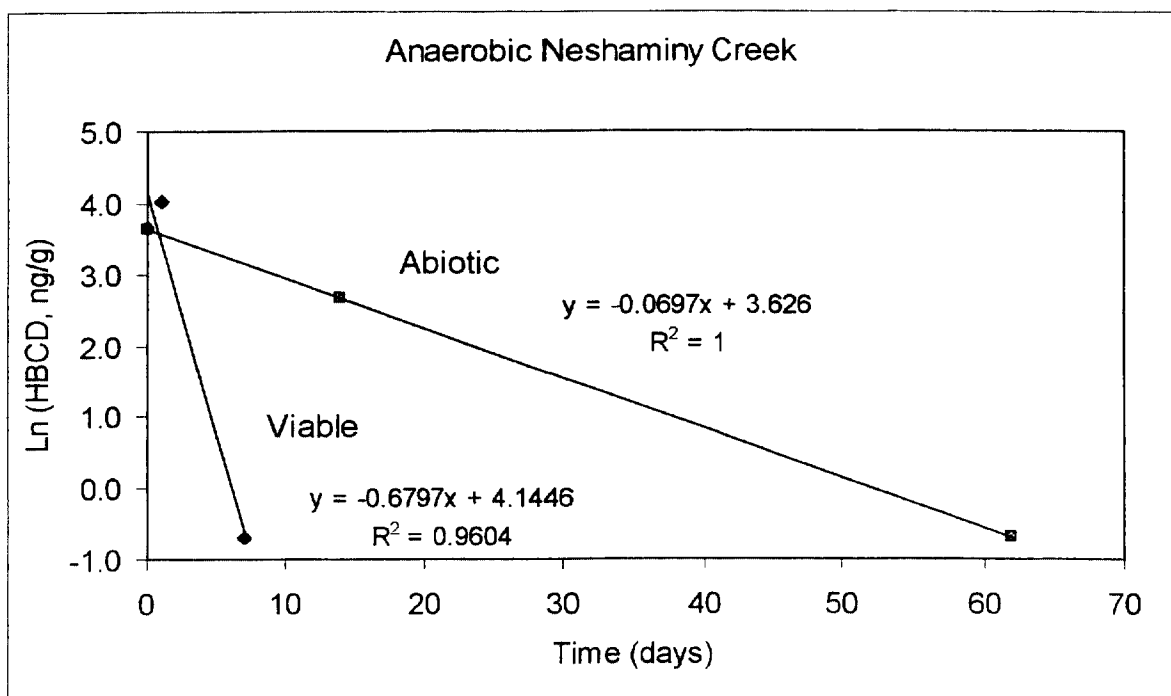
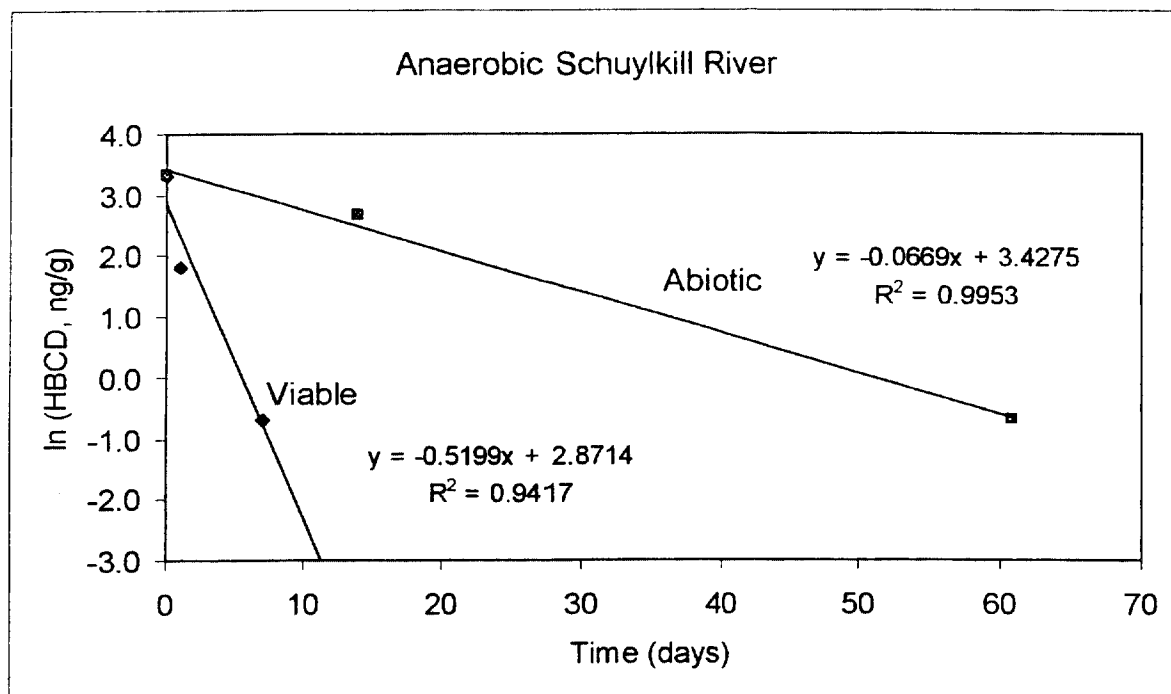
Flame Ionization Detector: 250 °C

Perkin-Elmer TurboChrom® Data Acquisition System

Appendix G. Plots of Natural Logarithms (HBCD, ng/g) versus Time for Aerobic Microcosms



Appendix H. Plots of Natural Logarithms (HBCD, ng/g) versus Time for Anaerobic Microcosms



Appendix I. Protocol and Protocol Change/Revision

Final Protocol

TOXICOLOGY & ENVIRONMENTAL RESEARCH AND CONSULTING
THE DOW CHEMICAL COMPANY

PROTOCOL

ENVIRONMENTAL CHEMISTRY RESEARCH LABORATORY
1803 BUILDING, MIDLAND, MICHIGAN 48674

TITLE: EVALUATION OF AEROBIC AND ANAEROBIC TRANSFORMATION OF
HEXABROMOCYCLODODECANE IN AQUATIC SEDIMENT SYSTEMS

KEY NUMBERS

FILE #: DR-0099-6827-003
STUDY ID: 021081
PTR: 60143-428-1
CAS: 25637-99-4

PLANNED DATES

EXPERIMENTAL START: June 24, 2002
EXPERIMENTAL TERMINATION: November 28, 2002
ESTIMATED FINAL REPORT: February 28, 2003
GLP STUDY: Yes

SPONSOR: American Chemistry Council's
Brominated Flame Retardant Industry Panel
1300 Wilson Boulevard
Arlington, Virginia 22209

REQUIRED SIGNATURES:

STUDY DIRECTOR: S. J. Gonsior 20 June 2002
S. J. Gonsior, M.S. /DATE

MANAGEMENT APPROVAL: K. A. Brooks 20 June 2002
K. A. Brooks, B.S. /DATE

SPONSOR or DESIGNEE: W. K. Sherman 21 June 2002
ACC Brominated Flame Retardant Industry Panel /DATE
Contact – Wendy Sherman

DISTRIBUTION – PERSONNEL LISTED ABOVE PLUS THE LIST BELOW

Albee, R. R., 1803 Bldg.
Burgert, L. C., 1803 Bldg.
Davis, J. W., 1803 Bldg.
Env. Chem. Office, 1803 Bldg.
Friederich, U., Horgen

Klecka, G. M. 1803 Bldg.
Rainey, M., 438 Bldg.
Stieve, B. L., 1803 Bldg.

OBJECTIVE

The objective of this study is to assess the biodegradability of hexabromocyclododecane (HBCD) under both aerobic and anaerobic conditions in aquatic sediments. The test procedure will be based on a modification of the Organisation for Economic Co-Operation and Development (OECD) proposed Guideline 308 "Aerobic and Anaerobic Transformation in Aquatic Sediment Systems" [1].

INTRODUCTION

HBCD is used as a flame retardant in several applications, including expanded and extruded polystyrene. HBCD has the potential for release into the environment from production processes, and from the processing and disposal of fabricated products containing the compound.

Biodegradation is a major process for removing chemicals from the environment. Biodegradation can take place under aerobic or anaerobic conditions. Aerobic biodegradation processes can predominate in surface waters, surface soils, and the aeration basins of wastewater treatment plants (WWTPs). Anaerobic processes, in contrast, can occur in aquatic sediments, groundwater, and anaerobic digestion units in WWTPs. Little information is available concerning the aerobic and anaerobic biodegradability of HBCD. The compound is reported not to be readily biodegradable based on the results of an OECD 301D Closed Bottle Test [2]. In this study, no degradation of 7.7 mg/L HBCD was observed in 28 days. Note that the test concentration of HBCD exceeded the reported water solubility of 3.4 µg/L by greater than 2000-fold. The lack of measurable biodegradation in this test was due, in part, to the limited availability of the test material to the microorganisms as well as the low amount of biomass contained in the inoculum.

Aerobic and anaerobic biodegradation studies will be conducted in microcosms prepared from aquatic sediments and associated waters. Concentrations of HBCD and any detected brominated degradation products will be determined in the water and sediment phases using a sensitive and compound-specific

analysis such as liquid chromatography/mass spectrometry (LC/MS). The analysis will allow the primary biodegradation of HBCD to be followed, and degradation rates to be determined.

MATERIALS

Test Material

A 25-gram sample of HBCD was obtained from Wildlife International, Ltd., Easton, Maryland. The sample was identified as a "Representative Sample of Composite of HBCD – WIL# 5850," with a lot number MC258500102. The sample is a composite of test materials supplied by Albemarle Corporation, Great Lakes Chemical Corporation, and Eurobom B.V. The sample has been characterized by Albemarle Corporation. Results of this analysis will be included in the study file and final report. The molecular formula for HBCD is $C_{12}H_{18}Br_6$ ($M_w = 641.7$). The vapor pressure of the compound is reported to be 6.27×10^{-5} Pa at 21 °C [2].

All other chemicals will be obtained from commercial sources, with appropriate documentation of identity and purity. Water will be purified in a MilliQ® Water Purification System (Millipore Corporation, New Bedford, Massachusetts).

Sediment and Water Samples

Wildlife International collected aquatic sediments and their associated waters for use in this study on April 23, 2002. Sediments were collected in both aerobic and anaerobic zones in freshwater systems (Schuylkill River, Valley Forge, Pennsylvania; Neshaminy Creek, Doylestown, Pennsylvania). Redox measurements were used to confirm the redox potentials of the sites. Additional measurements obtained during sample collection included temperature and pH. Samples were collected in glass bottles, packed with ice, and shipped via overnight air express to The Dow Chemical Company in Midland, Michigan. Upon receipt in the laboratory, the sediment and water samples were stored at approximately 4 °C. The sediment samples were passed through 2-mm sieves to remove stones, plant litter, and to improve sample homogeneity. The anaerobic sediments were processed in an anaerobic

glove box to minimize exposure to oxygen. The anaerobic glove box contains an atmosphere equilibrated from a gas mixture containing ~70% (vol) N₂, ~28% CO₂, and ~2% H₂.

Sub-samples of the sediments and water samples were sent to Agvise Labs (Northwood, North Dakota) for characterization. Sediment samples were characterized for texture (sand, silt, clay), pH, organic carbon content, cation exchange capacity, cations (Ca, Mg, Na, K, and H), bulk density, water holding capacity, total nitrogen, phosphorus, soluble salts and microbial biomass. Results for texture, percent organic carbon, and microbial biomass measurements are included in Table 1. Water samples were analyzed for pH, conductivity, alkalinity, cations (Ca, Mg, Na, K), carbonate, bicarbonate, nitrate, sulfate, chloride, turbidity, and total dissolved solids. Results of these characterizations will be included in the final report.

EXPERIMENTAL METHODS

Preparation of Microcosms

Reaction mixtures containing sediment and associated site water will be prepared in serum bottles as described in Table 2. The proportions of water and sediment necessary to meet guideline recommendations of water:sediment volume ratios between 3:1 and 4:1 and sediment layer thickness between 1 and 2.5 cm will be determined in preliminary experiments. The size of serum bottles used will be 250 mL. The serum bottles will be sealed with caps containing Teflon-coated rubber septa. Aerobic microcosms will be prepared on the laboratory benchtop. Anaerobic microcosms will be prepared and stored in an anaerobic glove box containing an atmosphere equilibrated from a gas mixture 70% (vol.) N₂, ~28% CO₂, and ~2% H₂.

The sealed reaction mixtures will be stored in the dark at approximately 20 °C for an acclimation period of one to four weeks to recover from the disturbance of sample collection or microcosm preparation, in accordance with guideline recommendations. The aerobic microcosms will be opened at least weekly and the headspace gases allowed to exchange with the air to ensure that aerobic conditions are maintained. Stability of both the aerobic and anaerobic microcosms, as reflected by

dissolved oxygen concentrations and pH measurements of the water phase, redox potentials of the water and sediment phases, and macroscopic separation of the water and sediment phases (visual observation) as appropriate, will determine the duration of the acclimation period. Detection of methane in the headspace of selected anaerobic microcosms may provide additional confirmation of anaerobic conditions. Resazurin, a redox dye will be added to a subset of anaerobic microcosms at 1 mg/kg to indicate when the redox potential of the reaction mixtures drops below -110 mV.

Following the acclimation period, the screw on caps for the aerobic microcosms will be removed and the headspace gases will be allowed to exchange with fresh air. HBCD will be added to replicate test mixtures at an approximate concentration of $10\text{ }\mu\text{g/kg}$ (based on sediment dry weight [3]). The reaction mixtures are described in Table 2. Acetone will be used as a carrier solvent to introduce the HBCD into the microcosms. The volume of carrier solvent used will be minimized to reduce the potential for inhibitory effects on the microorganisms present. [Note: If adequate analytical sensitivity for HBCD cannot be achieved following method development using the sediment matrices, the initial concentration of HBCD added to the reaction mixtures will be increased to ensure that the degradation/removal of HBCD can be followed through at least two half-lives in the sediment phase]. Blank mixtures and biologically inhibited control mixtures will be prepared to correct for possible analytical interferences and abiotic losses of the test material. Biologically inhibited controls will be prepared by heat sterilization of a subset of microcosms prior to the addition of the test material. Anaerobic microcosms will be spiked with HBCD in an anaerobic glove box to avoid exposure to oxygen.

Microcosm Incubation

Both aerobic and anaerobic microcosms will be incubated in the dark at 20 ± 2 °C. The concentration of oxygen in the headspace gases of selected aerobic microcosms will be monitored to ensure that aerobic conditions are maintained. If necessary, aerobic microcosms will be opened and the headspace gases exchanged with fresh air to help ensure that aerobic conditions are maintained. Microcosms will be sacrificed for analysis according to the schedule in Table 3. The sampling schedule may be

adjusted based on initial results. Note that the number of sampling days for the anaerobic microcosms is reduced from nine to seven days due to limited quantities of the anaerobic sediments that were available following processing (*i.e.*, sieving). The duration of the experiment will not exceed three months.

Analytical Methods

A liquid chromatography/mass spectrometry (LC/MS) analysis will be used to measure HBCD concentrations in this study. This methodology was previously developed for the analysis of HBCD in water and fish tissues, and will be modified and optimized for use in this study [4,5]. At selected incubation intervals, duplicate test mixtures will be sacrificed for extraction and analysis of HBCD (see Table 3). One blank mixture will be extracted and analyzed for potential background interferences, and a second blank mixture will be spiked with 10 µg/kg HBCD (based on sediment dry weight), extracted and analyzed for HBCD to determine the recovery of HBCD from the sample matrix. The water and sediment phases in the microcosms will be separated for extraction and analysis of HBCD. The extraction procedure will involve adding an immiscible organic solvent (*e.g.*, hexanes) to each phase, and shaking and/or sonicating the sample to facilitate extraction of sorbed HBCD into the organic solvent. When detected, brominated degradation products from HBCD will be tentatively identified and quantified by LC/MS. Gas chromatography/mass spectrometry (GC/MS) may also be used to identify brominated degradation products and evaluate possible interferences in the sample matrices. The headspace gases in selected microcosms will be analyzed to determine if volatile, brominated degradation products are formed. Analytical conditions will be included in the final report. Confirmation of identity and concentrations of the degradation products will likely depend on the availability of commercial standards. Sample extraction procedures, LC/MS and GC/MS conditions will be documented in the final report.

DATA AND REPORTING

Biodegradation of HBCD

Concentrations of HBCD and brominated degradation products that are detected in viable and biologically inhibited control reaction mixtures will be reported over time in both the water and sediment phases. Percent biodegradation (primary) for HBCD will be reported based on a comparison of measured HBCD concentrations at time t to HBCD concentrations measured at time 0. Loss of HBCD due to biodegradation will be determined by subtracting the rate of loss measured in biologically inhibited controls from the rate of loss measured in viable reaction mixtures.

The final report will contain information on the test material, sediment and water collection and characterization, test conditions, and interpretation of results as described in the test procedure [1].

Statistical Methods

Descriptive statistics (mean, standard deviation) will be used as appropriate to evaluate and report results from this study.

QUALITY ASSURANCE AND SAFETY

This study will be conducted in accordance with Good Laboratory Practice Standards [6,7,8,9]. The study conduct and data generated will be reviewed according to the procedures of the Quality Assurance Unit of Toxicology & Environmental Research and Consulting, The Dow Chemical Company. Permanent records of all data generated during the course of this study, the protocol, protocol revisions/changes, and the final report will be available for inspection by the Quality Assurance Unit of Toxicology & Environmental Research and Consulting, The Dow Chemical Company. All data generated including the protocol, protocol revisions/changes, and final report will be archived at the Toxicology & Environmental Research and Consulting, The Dow Chemical Company.

Laboratory procedures will be conducted in accordance with The Dow Chemical Company Research and Development EH&S Resource Manual. Routine safety precautions will include wearing gloves, safety glasses, and lab coats. In addition, specific safety information will be made available, and all personnel involved in the study will be advised of the safety precautions to follow prior to handling the test material and working in the laboratory.

REFERENCES

- 1.) Organisation for Economic Cooperation and Development Guidelines for Testing of Chemicals, Inherent Biodegradability (1992).
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- 9.) MHW/METI 59 KIKYOAU 85; EA, KANKIKEN No. 233; MHW, EISEI No. 38 and METI, 63 KIKYOAU No. 823.

Table 1. Characterization of Sediment Samples

Sample	Description	% Organic Carbon*	Classification (USDA)	% Sand	% Silt	% Clay	Microbial Biomass µg/g
Schuykill River	Aerobic	0.4	Sand	95	4	1	111
Schuykill River	Anaerobic	2.5	Loamy Sand	87	6	7	125
Neshaminy Creek	Aerobic	4.2	Sandy Loam	65	20	15	547
Neshaminy Creek	Anaerobic	4.2	Sandy Loam	74	20	6	218

*calculated from percent organic matter values determined from Walkley Black method

calculation: (% organic matter) x 0.58 = % organic carbon

Table 2. Description of Reaction Mixtures for Aerobic and Anaerobic Transformation Experiments in Aquatic Sediments

Reaction Mixture	Description	Analysis	Purpose
Blanks	Sediment/water mixtures + carrier solvent	LC/MS	Evaluate possible interferences in sample matrix
Test Mixtures	Sediment/water mixtures + 10 µg/kg HBCD (in carrier solvent)	LC/MS	Determine transformation rates of HBCD in water and sediment phases
Biologically Inhibited Blanks	Heat sterilized sediment/water mixtures + carrier solvent	LC/MS	Evaluate possible interferences in biologically inhibited sample matrix
Biologically Inhibited Control Mixtures	Heat sterilized sediment/water mixtures + 10 µg/kg HBCD (in carrier solvent)	LC/MS	Determine abiotic degradation rates for HBCD in water and sediment phases

Note: HBCD will be added to sediment mixtures at 10 µg/kg based on the sediment dry weight

Table 3. Sampling Schedule for Transformation Experiments in Aquatic Sediments

Day	Blanks*	Test Mixtures	Number of Microcosms Analyzed			Total number of Samples
			Biologically Inhibited Blanks*	Biologically Inhibited Controls		
0	2	2	2	2		8
1	2	2	-	-		4
7	2	2	-	-		4
14	2	2	2	2		8
21	2	2	-	-		4
28	2	2	2	2		8
56	2	2	-	-		4
90	2	2	-	-		4
120	2	2	2	2		8
Total:	18	18	8	8		52

For each sampling day, one blank mixture and one biologically inhibited blank mixture will be spiked with 10 µg/kg of HBCD (based on dry weight of sediment) to determine recovery of HBCD from sample matrix.

Note: Sampling schedules for anaerobic sediments will be reduced from nine to seven sampling days due to limited quantity of anaerobic sediments.

PROTOCOL CHANGE/REVISION

TOXICOLOGY & ENVIRONMENTAL RESEARCH AND CONSULTING THE DOW CHEMICAL COMPANY, 1803 BUILDING, MIDLAND, MICHIGAN 48674

Study Title: EVALUATION OF AEROBIC AND ANAEROBIC TRANSFORMATION OF
HEXABROMOCYCLODODECANE IN AQUATIC SEDIMENT SYSTEMS

Study ID: 021081

CHEC File Number: DR-0099-6827-003

Change(s)/Revision(s) Number: 1

CHANGE(S)/REVISION(S):

- 1.) Pre-incubation or stabilization period for aerobic microcosms was 6 to 7 weeks, rather than the range of 1 to 4 weeks described in the protocol.
- 2.) The volume ratios of the water and sediment layers in the microcosms ranged from 1.6 to 2.9. This is below the range of 3 to 4 described in the protocol.
- 3.) Measurements of oxygen concentrations in the headspace gases of the aerobic microcosms were used in place of dissolved oxygen measurements in the water layer.
- 4.) Redox measurements were made at the bottom of the water layer (at the water/sediment interface), rather than both the water and sediment layers as described in the protocol.
- 5.) Nominal HBCD concentration added to microcosms was increased from 10 ng/g (sediment dry weight) as described in the protocol to the range of 34 to 89 ng/g for the different sediments.
- 6.) The duration of the studies was 4 months (119 days), not the 3 months described in the protocol (page 6).
- 7.) The number of sampling days for the aerobic sediment studies was reduced from 9 to 7 sampling days.
- 8.) Matrix spikes were spiked with 34 to 89 ng/g of HBCD rather than the 10 ng/g described in the protocol.
- 9.) Beginning with samples collected on day 14 (anaerobic) or day 21 (aerobic), both background microcosms were typically spiked with HBCD as matrix spikes, rather than analyzed as single background and matrix spikes as described in the protocol.

REASON(S) FOR CHANGE(S)/REVISION(S):

- 1.) Additional time was required to develop and validate analytical method for HBCD.
- 2.) Target sediment thickness of 1.0 to 2.5 cm was met. The height of the water layer was decreased to 3.5 to 4.0 cm to maintain sufficient headspace in the 250-mL microcosms to ensure that sufficient oxygen was available in the aerobic microcosms.
- 3.) The headspace oxygen measurements used a fiber optic probe that did not disturb the water and sediment layers. This measurement allowed the oxygen in the the headspace gases to be replenished as necessary.
- 4.) Due to the small size of microcosms used, measurement of redox potentials within the sediment layer would have been impractical and would have severely disturbed the sediment layer.
- 5.) Higher HBCD concentrations were required to follow removal of HBCD through at least two half-lives, as noted in protocol.

- 6.) The 3-month value in the text of the protocol was incorrect. Table 2 in the protocol contained the correct value of 120 days. The OECD 308 guideline on which study was based recommends a 100-day maximum.
- 7.) The sampling schedule was adjusted based on the results of initial analyses. The revised schedule was sufficient to define the rate of removal of HBCD. The OECD guidelines recommend at least 6 sampling points.
- 8.) The concentration of HBCD added to the matrix spikes was increased to match the higher initial dosing of the test microcosms.
- 9.) Analysis of two matrix spikes on a sampling day provided improved precision for quantitation of HBCD concentrations in the microcosms.

IMPACT ON STUDY:

1-9) None

PLEASE FILE THIS CHANGE/REVISION WITH THE PROTOCOL.

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